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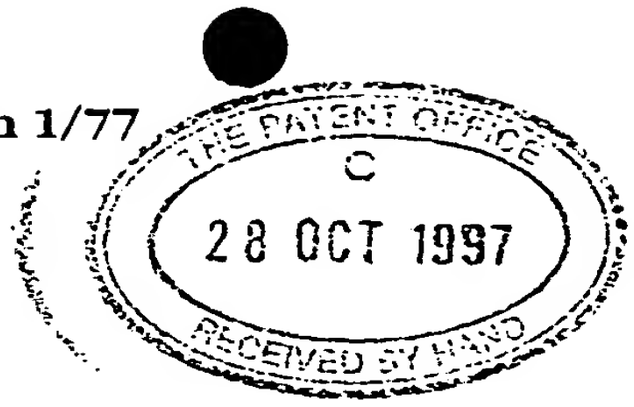

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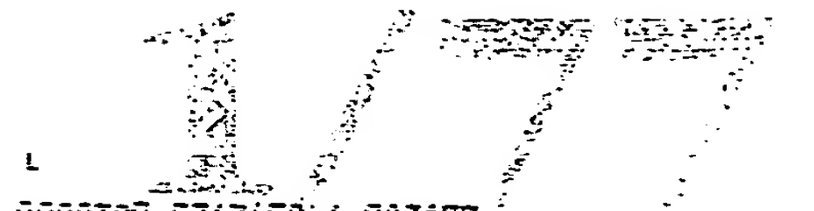

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ISIS INNOVATION LIMITED
2 South Parks Road
OXFORD
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3998564001

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — UNITED KINGDOM

4. Title of the invention — HUMAN CD8 AS AN INHIBITOR OF THE CELLULAR IMMUNE SYSTEM

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

STEVENS HEWLETT & PERKINS
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HUMAN CD8 AS AN INHIBITOR OF THE CELLULAR IMMUNE SYSTEM

INTRODUCTION

Major histocompatibility complex Class I and II proteins (MHC, or HLA in man) bind peptide antigens and present them on the cell surface for recognition by T lymphocytes expressing a unique T cell receptor (TCR) matching the specific MHC-peptide combination. The transmembrane glycoproteins CD8 and CD4 are characteristic of distinct populations of T lymphocytes whose antigen responses are restricted by class I and class II MHC molecules, respectively. CD8 and CD4 play major roles both in the differentiation and selection of T cells during thymic development and in the activation of mature T lymphocytes in response to antigen presenting cells. CD8 and CD4 are therefore considered to be the main accessory molecules for T cell receptors. Although CD8 and CD4 are immunoglobulin superfamily proteins and determine antigen restriction by binding to MHC molecules, but not to the antigenic peptide, the structural basis for their similar functions appears to be very different. Their sequence similarity is low and whereas CD4 is expressed on the cell surface as a monomer CD8 is expressed as an $\alpha\alpha$ homodimer or an $\alpha\beta$ heterodimer.

CD8 expression is characteristic of cytotoxic T lymphocytes (CTL) and important for their progression through the process of 'positive selection' during differentiation in the thymus. In mature cytotoxic lymphocytes the immunological role of CD8 is complex and, as yet, not fully clarified. From the current understanding three putative roles in CTL function can be envisaged, CD8 plays a role in T cell signalling. Secondly, a significant function of CD8 may be to assist in cell-cell adhesion tethering the T cell to the antigen presenting cell by increasing the avidity of the interaction. In most, but not all, mature cytotoxic lymphocytes target cell

lysis can be blocked by anti-CD8 antibodies. Thirdly, CD8 may serve as a coreceptor for TCR involving a mechanism for collaborative binding of the two receptors to the MHC/peptide ligand. Comparison of the structures of the TCR-MHC and CD8-MHC complexes provide clues for how this cooperativity could work. Although CD8 imposes no detectable conformational changes on the peptide loaded cleft of the MHC molecule, where the TCR binds, it does introduce a shift in a3 domain which is similar to a shift introduced by TCR binding. Thus, binding of either CD8 or TCR to the MHC complex may energetically favour binding of the other.

The T cell coreceptor CD8 is essential for the positive selection of cytotoxic T-lymphocytes (CTL) during differentiation in the thymus and for the ability of most mature CTLs to kill target cells. CD8, expressed on the T cell surface as $\alpha\alpha$ or $\alpha\beta$ dimers, contacts the MHC class I molecules on antigen presenting cells, increasing the avidity of the T cell for its target. CD8 is also involved in the phosphorylation events leading to T cell activation through the association of the a chain cytoplasmic tail with p56lck.

The analysis of the molecular basis underlying cellular immune responses have suffered from the lack of availability of soluble protein reagents with which to study structural and kinetic aspects of the interactions involved. Recently however a number of groups using expression systems ranging from E.coli to mammalian cells have reported strategies for obtaining soluble TCR.

THE INVENTION

A strategy for overexpression in *E.coli* of the extracellular immunoglobulin domain of human CD8α was devised using codon usage alterations in the 5' region of the gene, designed so as to prevent the formation of secondary structures in the mRNA. A fragment of CD8α, comprising residues 1-120 of the mature protein thus excluding the signal

peptide and the membrane-proximal stalk region, was recovered from bacterial inclusion bodies and refolded to produce a single species of homodimeric, soluble receptor. Figure 2 illustrates the domain structure of CD8 as described above, and showing other truncations of the CD8α protein which were expressed but did not fold correctly. A2 heavy chain and β2-microglobulin were similarly expressed and refolded with a synthetic peptide antigen corresponding to the *pol* epitope from HIV-1. CD8α/HLA-A2 complexes were formed in solution and by co-crystallization with a stoichiometry of one CD8αα dimer to one HLA-A2-peptide unit.

Data is herein given demonstrating that a fragment of the CD8αα receptor, corresponding to the immunoglobulin-like domain plus a short fragment of the membrane-proximal stalk region, is capable of inhibiting T cell cytotoxicity, and that mutations of that CDαα fragment can modulate that capability. This discovery forms a major part of the invention. Also described herein is the above strategy for high-level expression of the extracellular immunoglobulin domain of human CD8α in E.coli and for the folding of the CD8αα dimer as a MHC-binding receptor. Also described is the assembly and crystallisation of the complex between CD8αα and HLA-A2 associated with peptide antigen.

Thus the invention provides in one aspect, a protein having the sequence shown in figure 1, said protein folded as a dimer and having the property of inhibiting the action of cytotoxic T cell lymphocytes to kill target cells.

In a second aspect the invention provides a protein which differs from that defined above, in one or more of the following respects: i) methionine present at the N-terminus; ii) one or a few amino acid residues absent from the N-terminus; iii) one or a few amino acid residues added at the N-terminus consisting of part or all of the sequence 'leu - leu - leu - his - ala - ala - arg - pro -'; iv) one or a few amino acid residues absent from the C-terminus; v) one or a few amino acid residues added at

the C-terminus consisting of part or all of the sequence '- ala - pro - arg - pro - pro -thr - pro - ala'; vi) part of all of the CD8 cytoplasmic membrane peptide sequence added at the C-terminus; vii) conservative variants of one or many amino acid residues which do not materially affect the CD8 functionality of the protein; viii) mutations which do alter the CD8 functionality, including those which alter or abolish or reverse the property of inhibiting the action of cytotoxic T cell lymphocytes to kill target cells; ix) the addition of a protein or peptide, at the N or C terminus for the purposes of purification; x) the provision of a label for detection; said protein folded as a dimer being soluble having the property of affecting the action of cytotoxic T cell lymphocytes to kill target cells. Particularly provided is a protein as described above, in which E replaces N at position 99. Also provided is a protein as described above, in which EE replaces QN at position 54, 55.

In another embodiment, the invention provides a complex of a protein as herein defined with HLA - A2. A further form of this complex is provided in crystalline form with a stoichiometry of one protein dimer to one HLA - A2 peptide unit.

In a further embodiment, the invention provides a method of preparing a formulation for affecting the immune system of a patient, which method comprises bringing a protein as herein defined into a form suitable for administration.

The invention provides, in a still further embodiment, a method of producing a recombinant protein as herein described, which method comprises the steps of: i) providing a CD8 derived gene suitably modified to allow expression of a protein, essentially corresponding to at least the immunoglobulin-like domain of a CD8 protein, in a bacterium; ii) effecting expression of said CD8 derived gene in said bacterium and recovering of the expressed protein from a bacterial culture; iii) treating the expressed protein to facilitate its purification and carrying out said

purification. Preferably at step i) the CD8 derived gene is modified via silent mutations designed to increase expression via the prevention of the formation of a 5' hairpin secondary structure in the expressed mRNA. Preferably at step iii) the treatment of the expressed protein involves solubilising the protein and treating the protein so as to cause it to fold into a form resembling its native state, which is then purified. Preferably the CD8 derived gene product corresponds to the immunoglobulin-like and membrane-proximal stalk regions of a CD8 protein.

The invention provides, in a still further embodiment, the use of the protein as herein defined as a means of modulating an immune response via contacting said protein with cytotoxic T lymphocytes. In particular the invention provides a use of the CD8 derived protein, as a means of inhibiting the immune response of cytotoxic T lymphocytes.

1. Structure of the CD8αα receptor.

The human CD8 gene expresses a protein of 235 amino acids. As illustrated in figure 2, the organisation of the protein can be divided into the following domains (starting at the amino terminal and ending at the carboxy terminal of the polypeptide):

a. signal peptide (amino acids -21 to -1) - this is cleaved off in human cells during the transport of the receptor to the cell surface and thus does not constitute part of the mature, active receptor;

b. immunoglobulin (Ig) -like domain (approximately amino acids 1-115) - this domain assumes a structure, referred to as the immunoglobulin fold, which is similar to those of many other molecules involved in regulating the immune system, the immunoglobulin family of proteins. The crystal structure of the CD8αα receptor in complex with the human MHC molecule HLA-A2 has demonstrated how the Ig domain of CD8αα receptor binds the ligand;

c. membrane proximal stalk region (amino acids 116-160). This

domain is thought to be an extended linker region allowing the CD8αα receptor to ' reach' from the surface of the T-cell over the top of the MHC complex to the α3 domain of this where it binds. The stalk region is glycosylated and thought to be inflexible. In the context of this invention it should be noted that the stalk region is assumed to play no part in MHC binding;

d. transmembrane domain (amino acids 161-188). The transmembrane domain anchors the CD8aa receptor in the cell membrane and is therefore not part of the soluble recombinant protein;

e. cytoplasmic domain (amino acids 189-214). The cytoplasmic tail of CD8 mediates a signalling function in T-cells through its association with p56lck which is involved in the T cell activation cascade of phosphorylation events.

The invention encompasses two forms of the CD8 receptor, αα and αβ (EMBL/GENBANK database accession numbers: CD8α, M27161; CD8β, X13444). The two forms of the receptor are functionally equivalent and no significant differences in the effects of using one or the other for immune inhibition would be expected.

The essential feature of any soluble CD8 protein envisaged is that it contains a correctly folded Ig domain since it is this region that constitutes the binding region for contacting MHC molecules and therefore is responsible for the modulating effect. Variants of the CD8 proteins within the scope of the invention are envisaged and are listed as follows:

a. Variations of the C-terminal truncation point. It is possible that longer or shorter versions of the receptor could also be stable and functional. There is no general rule to predict where the optimal truncation point for a soluble version of a transmembrane protein is. In the case of CD8, the polypeptide could be between 1 and 15 amino acids longer or shorter, or it could even comprise the cytoplasmic domain having just the transmembrane domain deleted. It is also envisaged that the C-terminus

could be fused to peptides or protein domains, such as glutathione-S-transferase for purification purposes, as well known in the art.

b. Variations in the N-terminal truncation point. There is some uncertainty as to where exactly the signal sequence ends and the Ig domain starts. The N-terminal truncation point could probably be varied somewhat, just like the C-terminal truncation point, without any influence on the functional effect of the protein. It is also envisaged that the N-terminus could be fused to peptides or protein domains, such as glutathione-S-transferase for purification purposes, as well known in the art.

c. Amino acid substitutions. A large number of conservative amino acid substitutions can presumably be introduced in the protein without causing any significant change in the binding between CD8 receptor and MHC.

d. Mutations designed to alter functionality. With the structure of the complex between CD8αα receptor and a MHC molecule, HLA-A2, available to the inventors, it is now possible for them to design mutations predicted to affect the interaction and therefore the immuno-inhibitory effect. The testing of two such mutations is described below, the substitution of residue 99 by a glutamic acid (designated ' 99E') and the double substitution of residues 54 and 55 by glutamic acids (designated '54,55E'). These mutations were designed as controls, i.e. the mutations were intended to abolish the binding of the CD8αα receptor to MHC. Mutant 99E has a residual effect while mutant 54, 55E shows no sign of inhibition (see following T cell lysis data contained below). Thus the design of mutations with the opposite effect, i.e. which increase the binding between CD8αα receptor and MHC molecules is also envisaged.

The examples that follow are more clearly described with reference to the following figures:

Figure 1 shows the amino acid sequence of a soluble recombinant CD8α protein.

Figure 2 shows CD8α and truncated proteins expressed in *E.coli*. Schematic presentation of CD8α illustrating domain organization (top) and the extent of the truncated proteins expressed in bacteria (below). L = leader sequence, Ig = immunoglobulin domain, MP = membrane proximal domain, Tm = transmembrane domain, Cyt. = cytoplasmic domain. Numbers indicate amino acid positions of domain boundaries in relation to the mature protein and the endpoints of proteins expressed in *E.coli*. 'C' signifies the presence and positions of the cysteine residues at positions 143 and 160 involved in forming the interchain disulfide bonds in the CD8αα dimer. Expression vectors containing the codon information for the illustrated truncated proteins were constructed both with the original codon usage for human CD8α and with codon usage changes in the 5' end of the insert as described in Experimental Procedures.

Figure 3 shows expression and purification of CD8α. Approximately 10µg protein samples were analyzed by separation on a 15% SDS-PAGE gel. M = protein size marker; lane 1 = whole lysate of uninduced BL21 cells containing the expression plasmid for residues 1-120 of CD8α with original codon usage; lane 2 = as lane 1 but induced with 0.5 mM IPTG for 4 hours; lane 3 = lysate of uninduced BL21 cells containing CD8α expression plasmid with altered codon usage at the 5' end; lane 4 = as lane 3 but induced with 0.5 mM IPTG for 4 hours; lane 5 = purified inclusion body fraction from cells as in lane 4; lane 6 = refolded and purified by gel filtration and ion exchange. The molecular weight of the size markers and the expected position of CD8α are indicated.

Figure 4 shows purification of CD8α. *A*, Superdex G-75 gel filtration profile of concentrated CD8α refolding. Numbers over the profile indicate the elution points of molecular weight standards. *B*, NaCl elution profile from Mono-S column of the main peak from the gel filtration.

Figure 5 shows electrospray mass spectrometry analysis of

soluble CD8αα receptor. *A*, electrospray mass spectrum of CD8αα showing mass-to charge ratio peaks after proton bombardment; *B*, deconvolution of the spectrum shown in *A* indicating the molecular weight of the single dominant peak.

Figure 6 shows co-refolding fel filtration profile of CD8α and HLA-A2. Gel filtration was performed on a Superdex G-200 column. Numbers over the profile indicate the elution points of molecular weight standards. The protein components of the obtained peaks are indicated over the arrows.

Figure 7 shows SDS-PAGE analysis of co-refolded and co-crystallized CD8αα and HLA-A2. Lane 1-4 and M were stained with Coomassie Blue, lane 5 was silver stained. Lane 1 and 5: co-refolded CD8α, HLA-A2, β2m and peptide from peak indicated in Figure 5; lane 2: resolubilized crystal; lane 3: HLA-A2; lane 4: CD8αα, M: protein size markers as indicated. The bands corresponding to A2 heavy chain (A2 HC), β2m and CD8α are indicated with arrows.

Figures 8 to 15 are discussed in detail in example 2.

EXAMPLES

1. High level expression of CD8αα homodimer in *E. coli and crystallisation.*

MATERIALS AND METHODS

Cloning of CD8α cDNA and Construction of E.coli Expression Vectors -

A CD8α encoding DNA fragment was obtained by the polymerase chain reaction (PCR) using cloned Pfu polymerase (Stratagene) and cytotoxic lymphocyte cDNA as template. The PCR reaction was performed with the sense primer:

5'-GACTGAGTCGCGGCCGCTGCCACCATGGCCTTACCAGTGACCGCC
TTG-3';
and the antisense primer:
5'-TATTCGACTGGATCCTTATACGTATCTCGCCGAAAGGCTGGG-3'.

The PCR product was restriction digested with Eag I and BamHI and cloned into pBluescript II KS- (Stratagene) to obtain plasmid BJ082. The presence of the full length CD8α coding sequence was verified by doublestranded dideoxy sequencing (USB 70754) using T7 polymerase (Pharmacia). For the construction of bacterial expression vectors five PCR products using BJ082 as template were generated which encode a methionine followed by the mature immunoglobulin like domain of CD8α. The PCR reactions were conducted with the sense primer
5'-GGAATTCCATATGAGCCAGTTCCGGGTGTCGCCGCTGGATCG-3'
and the antisense primers:
A:
5'-CGCGGATCCCTATGCGCCCCCCGCTGGCCGGCTCGCCTCTGGGC
GCAGGGACAG-3';
B:
5'-CACCGCGAATTCGGATCCTAAGCGGGTCTACAAGCTTCGGGCTTC
GCTGGCAGGAAGACC-3';
C:
5'-CACCGCGAATTCGGATCCTAAGCGGGTCTACAAGCTTCTGGCGTC
GTGGTGGGCTTCGC-3';
D:
5'-CACCGCGAATTCGGATCCTAAGCGGGTCTACAAGCTTCGGGCTTC
GCTGGCAGGAAGACC-3';
or **E:**
5'-GTGGCAAGCTTGGATCCTATGGCGTCGTGGTGGGCTTCGCTG-3'.

These PCR products were restriction digested with Nde I and Bam HI and cloned in vector pGMT7 to obtain expression cassettes for

residues A: 1-150 with the cysteine residue at position 143 mutated to serine, B: 1-146, C: 1-120/141-146, D: 1-116/141-146, and E: 1-120. After sequence verification the vectors were tested for expression (see below) but induction of CD8α protein expression from all vectors was only detectable by immunoblot analysis employing the monoclonal antibody OKT-8 (ATCC reference CRL8014). Five new PCR fragments encoding the corresponding proteins were subsequently generated using a modified sense primer,

5'-GGAATTCCATATGAGTCAATTTCGTGTATCACCGCTGGATCG-3' ,

which introduces six silent mutations in the coding region. These PCR fragments were restriction digested with *Nde* I and *Hind* III and cloned into the vector pGMT7 to obtain plasmids UG195 (A), UG231 (B), UG222 (C), pAÆ3 (D), and BJ112 (E).

Construction of HLA-A2 Expression Vector -

A DNA fragment encoding the a1, a2, and a3 domains of HLA-A*0201 (HLA-A2), comprising residues 1-276, was obtained by PCR using B cell cDNA as template. The PCR reaction employed the sense primer: 5'-ACATACCCATGGGCTCTCACTCCATGAGGTATTTC-3';

and the antisense primer:

5'-ACATACAAGCTTACGGCTCCCATCTTAAGGTGAGGGGCTTGGG-3'.

The PCR product was restriction digested with *Nco* I and *Hind* III and cloned in pET23d+ (Novagen) to obtain plasmid BJ075a in which the sequence of the HLA-A2 fragment was verified by dideoxy sequencing.

The plasmid pHN1- β2m used for expression of the β2 microglobulin subunit was kindly provided by David N. Garboczi and Don C. Wiley, Harvard Medical School.

Expression of CD8α in E.coli -

T7-CD8α expression vectors were transformed into the *E.coli*

strain BL21DE3pLysS (Novagen) and from single colonies cultures were grown in TYP medium containing 100 µg/ml Ampicillin. Expression from the T7 promoter was induced at approximately OD600 = 0.4 by the addition of IPTG to a final concentration of 0.5 mM and the time-course of induction initially followed for up to eight hours. No increase in protein yields were observed with any constructs after 4 hours following induction by IPTG.

Large Scale Preparation and Solubilization of CD8α from Bacterial Inclusion Bodies -

Bacterial cells were recovered by centrifugation and resuspended in Lysis Buffer (20 ml per liter original bacterial culture) containing 10 mM EDTA, 2 mM DTT, 10mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% v/v Glycerol, 200 mg/l Lysozyme, 1.0mM PMSF. The solution was freeze-thawed and sonicated (Misonix W38S) for a total of 5 min. After mixing with an equal volume of Wash Buffer (0.5% v/v Triton X-100, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM DTT, 0.1% v/v Sodium azide) the solution was left on ice for a further 30 min. Inclusion bodies were pelleted by centrifugation at 15,000 rpm in a Beckman JA-20 rotor for 10 min., then resuspended in Wash Buffer (20 ml per liter original culture) using a teflon homogeniser. The recovery and wash steps were repeated four times in total. Following the last spin the pellet was solubilized in Urea Buffer made up as 8 M Urea, 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 10% v/v Glycerol, 500 mM NaCl, 10 mM DTT and end-over-end mixed overnight at 4°C. Debris was cleared from the inclusion body suspension by centrifugation as described above for 30 min. and the cleared suspension stored at -70°C.

Refolding of the CD8αα Receptor -

The CD8α protein (at 10 mg/ml) in 2 ml Urea Buffer was

added to 15 ml Guanidine Buffer (6 M Guanidine hydrochloride, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl, 10 mM DTT) and allowed to equilibrate at room temperature for 30 min., then added to 1 litre Refolding Buffer made up as 200 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1 M L-arginine, 0.1 mM PMSF, 6.5 mM Cysteamine and 3.7 mM Cystamine. Two more pulses of 20 mg protein were added to the refolding mixture at 24 hour intervals and the solution stirred at 4°C for 72 hours in total.

Refolding of HLA-A2/pol -

HLA-A2 heavy chain, β2m and peptide (ILKEPVHGV, synthesized by Oxford Centre for Molecular Sciences and corresponding to residues 309-317 of HIV-1 reverse transcriptase) were refolded essentially as described with the modification that Guanidine Buffer was added to the protein before injecting this into Refolding Buffer as described above for folding of CD8α (David N. Garboczi, personal communication).

Purification of Folded CD8αα Receptor -

The refolding mixture was cleared by centrifugation for 30 min. at 4,000 rpm in a Beckman J-6B, after which the supernatant was concentrated to approximately 200 ml in an Amicon 2000 Stirred Cell using a Diaflow ultrafiltration membrane with 10 kD pore exclusion size. Following this the sample was centrifuged for 30 min. at 15,000 rpm in a Beckman JA-20 rotor, then further concentrated to 10 ml in an Amicon 8400 Stirred Cell. Prior to a two-step purification procedure involving gel filtration and cation exchange the concentrated sample was filtered through a 0.22 µm sterile filter. Subsequent preparations of CD8α have indicated that the centrifugation and filtration steps introduced to clear the protein solution between refolding and concentrating are essential for maintaining correctly folded receptor in solution. The gel filtration step was performed on a Pharmacia Superdex G-75 column equilibrated in 20 mM Tris-HCl (pH

8.0), 150 mM NaCl. Fractions containing CD8αα homodimer were collected and buffer exchanged into 50 mM MES (pH 6.5) using an Amicon Centriprep-10. The ion exchange step was performed on a Pharmacia Mono-S column from which the protein was eluted with a gradient of 0-1 M NaCl in 50 mM MES (pH 6.5).

Biochemical and Physical Analysis of Purified Proteins -

Protein concentrations of the folded and purified proteins were determined by Bradford Assay (BioRad). The identity of the folded CD8α was confirmed by Edman sequencing of the N-terminus. Mass spectroscopic analysis of CD8αα was performed using a VG Instruments triple-quadropole atmospheric mass spectrometer fitted with an electrospray ionisation interface. Protein samples of 10 µg in 20 µl 1:1 v/v acetonitrile/water, acidified by addition of formic acid to a final concentration of 1% v/v, were injected at a flow rate of 20 µl/min. The mass spectrometer was calibrated using horse heart myoglobin (Mr 16951.48).

Crystallisation of the CD8αα /HLA-A2 Complex -

1 µl protein solution containing CD8αα at 10 mg/ml and HLA-A2/pol at 20 mg/ml were mixed with 1 µl reservoir solution from Crystal Screen kits I and II (Hampton Research). Crystallization trials were performed by vapour diffusion from sitting drops on microbridges at room temperature.

RESULTS

Mutations that Decrease the Tendency for Secondary mRNA Structures in the 5' end of the CD8α Gene Greatly Increase the Expression Potential in E.coli -

To construct CD8α expression vectors for use in a range of expression systems a DNA fragment constituting the entire open reading frame was first generated by the polymerase chain reaction (PCR) using cDNA prepared from a cytotoxic T-lymphocyte cell line as template. A plasmid containing this fragment was then used as template in a second generation of PCR reactions to synthesize five CD8α cDNA fragments which were inserted in plasmids containing the T7 promoter. These constructs all encode polypeptide chains in which an initiation codon is followed by the mature CD8α N-terminal region, comprising the entire immunoglobulin domain but truncated at different points C-terminally of this (Fig. 2). Constructs with equivalent C-terminal truncations but incorporating the CD8α signal sequence were tested for expression from a baculovirus promoter in insect cells.

Previously, expression of human CD8α in insect and CHO cells has indicated that disulfide bonding of the dimer through Cys143 is critical for the production of a secreted receptor. In contrast, Cys160 which is also involved in covalently linking the membrane-bound receptor was found to prevent expression of soluble CD8αα. In accordance with this we found that in insect cells CD8α 1-146, 1-120/141-146 and 1-116/141-146 could be expressed as secreted dimers (data not shown). The requirement for the Cys143 residue for secretion of CD8αα dimer from insect cells indicates that this residue is essential either for the folding of the protein or for its stability during intracellular transport. These possibilities can not at present be distinguished and since cysteine residues pose a potential problem when refolding protein in vitro constructs incorporating or omitting Cys143 were tested in bacteria (Fig. 2).

From plasmids containing inserts with the original human CD8α codon usage no induction of expression could be detected in *E.coli* as assessed by comparison of whole cell extracts after gel electrophoresis and coomassie staining (Fig. 3, lanes 1-2). In contrast, expression was

readily detectable when six codons in the CD8α gene were altered by silent mutations to make the 5' end of the gene more A/T rich (Fig. 3, lanes 3-4). It has previously been reported that such changes, as would be expected with a lower G/C content and therefore weaker base-pairing potential, decrease the tendency for hairpin formation in the 5' end of the CD8α mRNA. It appears likely, therefore, that secondary structure formation in the original sequence has a severe adverse effect on the potential for expression in *E. coli*. Expression from the mutated constructs are estimated to yield in excess of 100 mg per litre, an increment of at least two orders of magnitude over the expression level obtained with the unmutated sequence.

Large-scale Preparation, Initial Purification and Solubilization of Bacterially Expressed Proteins -

Analysis of cellular fractions from lysed bacteria expressing CD8α showed that virtually all the protein is deposited in inclusion bodies (Fig. 3) as has previously been observed with bacterally expressed MHC heavy chain and β2m polypeptides. Purification of the CD8α inclusion bodies by washing yielded protein estimated to be approximately 90% pure (Fig. 3, lane 5). Similar inclusion body purity was obtained with HLA-A2 and β2m (not shown). CD8α inclusion bodies were not soluble in the pH 6.5 buffer used for the MHC chains but adjustment of pH to 8.0, high salt and the addition of 10% Glycerol made it readily soluble.

Refolding and Quality Analysis of Soluble CD8αα Receptor -

The resolubilized CD8α polypeptide in reducing and denaturing buffer was diluted into conditions permitting formation of native protein conformation. After allowing folding to proceed 48 hours soluble protein was concentrated for analysis and purification by gel filtration. Four of the polypeptides expressed and illustrated in Fig. 2 produced mainly

aggregate peaks of high molecular weight (data not shown). In contrast to this the elution profile of the 1-120 polypeptide shows one major peak of approximately 30 kDa as expected for the CD8αα dimer (Fig. 4A). In this elution profile no indication of aggregates is evident. The CD8αα dimer was further purified by cation exchange on a Mono-S column eluting as a single peak at approximately 175 mM NaCl (Fig 4B). Gel analysis of this peak shows only one band at the expected position for CD8α (Fig. 3, lane 6).

The identity of the purified CD8α was verified by two methods. Edman sequencing produced a run of ten amino acids corresponding to the mature N-terminus of CD8α showing that the introduced methionine residue had been cleaved off in *E.coli*. Electrospray mass spectrometry confirmed the molecular weight of CD8α (13,464.0) as very close to the theoretical value of 13,463.2 Dalton for residues 1-120 excluding the initiating methionine (Fig. 5). A smaller peak of slightly higher molecular weight is likely to correspond to protein that has not had the methionine residue cleaved off (Fig. 5).

Co-refolding and Soluble Complex Formation between CD8αα Dimer and HLA-A2/pol -

To test whether the efficiency of refolding of the component molecules could be increased by complex formation a co-refolding experiment was performed. Gel filtration analysis of the concentrated protein reveals that the folding of both CD8αα and HLA-A2 are impaired and that more aggregates are formed under these conditions than during individual refolding (Fig. 6). A peak is detectable, however, at the expected position corresponding to approximately 75 kDa for the CD8αα /HLA-A2 complex which is not present in neither the CD8α refolding (Fig. 4A) nor in the HLA-A2 refolding (not shown). Analysis of the protein in this peak shows that the components expected in the complex are

present in amounts corresponding to one CD8αα dimer to one HLA-A2 (Fig. 7, lane 1 and 5) indicating that stable complex formation can take place in solution.

Crystallization of CD8αα in Complex with HLA-A2/pol -

Crystallization trials, performed with a 2:1 mixture of separately refolded HLA-A2 and CD8αα, yielded visible crystals under two buffer conditions, 12% PEG 20,000, 100 mM MES pH 6.5 and 15% PEG 6000, 50 mM MES pH 6.0, after approximately seven days at room temperature. One crystal was later diffracted at 2.7 Å resolution. To analyze the composition of the crystal a fragment was washed, resolubilised and analysed by gel electrophoresis (Fig. 7, lane 2). The protein bands present in this sample correspond exactly to those from the individual HLA-A2 and CD8α (Fig 7, lanes 3 and 4, respectively). Furthermore, the ratio of the CD8α to the heavy chain and β2m bands are identical to those found in the sample from the co-refolding peak thought to correspond to the receptor -ligand complex (Fig. 7, lane 1).

DISCUSSION

Expression of CD8α

For expression of CD8α we found the yields obtained from insect cells insufficient for large-scale purification and therefore focussed on expression in *E.coli*. In bacteria three parameters generally appear to be the main determinants of the expression level which can be achieved for a foreign protein. These are the tendency for secondary structure in the mRNA, the degree to which the codon usage conforms to bacterial preference, and the toxicity for the host of the expressed protein. In the case of CD8α, six silent codon changes, designed to decrease base-pairing potential close to the N-terminus, increased the expression level by more than two orders of magnitude to a level constituting approximately 30% of the total protein. The high expression levels and the ease with

which the protein can be purified from inclusion bodies made bacterial expression the preferred choice for further studies.

Refolding of CD8α

The refolding efficiency of CD8α was found to depend crucially on the buffer conditions used to solubilise the protein from inclusion bodies. In particular the maintenance of a high redox potential, high ion strength and the addition of strongly denaturing agents before refolding dramatically improves the yield of the CD8αα dimer. Analysis of the folded receptor preparation by gel filtration shows virtually no presence of aggregates and after cation exchange the preparation appears to be completely homogeneous and stable for several months at 4°C.

Analysis of the CD8αα-HLA-A2 Complex Formed in Solution and by Crystallization

The estimated molar ratios of the polypeptide bands representing HLA-A2 assembly and CD8αα are identical in the complex formed in solution and in the crystal but the stoichiometry indicated by the band intensities is somewhat unexpected. Because CD8 has been detected on the T cell surface as a dimer it has been speculated that CD8 would bind two MHC molecules and that a bivalent stoichiometry could be involved in T cell-target cell ligation and T cell activation. However, the gel analysis of the complexes, taking into consideration that CD8a stains weaker with coomassie than does β2m, are more indicative of a 1:1 HLA-A2:CD8aa ratio and this has been confirmed by the crystal structure. Based on the crystal structure steric considerations rule out that more than one CD8aa dimer can bind the MHC.

Recent studies involving cells or soluble murine CD8 and TCR have indicated that a mechanism for cooperative binding to class I MHC may exist for the two receptors. Comparison of the crystal structures

of human CD8aa/HLA and TCR/HLA indicate that both receptors introduce a shift in the a3 domain of the HLA heavy chain which could be associated with an energetic penalty upon binding. Further studies on complexes of MHC Class I with human CD8 and TCR should clarify whether the structural shift introduced in the HLA heavy chain constitutes a mechanism for cooperative binding of these receptors.

Example 2. Immune-inhibitory effect of the CD8αα receptor.

In a standard cytotoxic T cell assay suitable target cells, i.e. cells which express the relevant restrictive MHC, are first incubated with peptide and radioactive chromium. After washing the target cells are exposed to T cells. The T cell, through T cell receptor (TCR) and CD8 recognises, the complex of the peptide and MHC on the target cell surface and, if the ligand is appropriate and present in sufficient concentration, the T cell will lyse the target cell. Lysis is assessed by measuring the release of radioactive chromium.

Figure 8 shows the results of a series of experiments conducted as follows:

- A human T cell line (enriched and selected), recognising the HIV1 'gag' peptide restricted by HLA-A2, was used.

- A constant number of target cells, 5000, '868' B cells, were used and the number of T cells was varied. 'E:T' represents a ratio of numbers of T cells to target cells. Four E:T ratios were tested.

- The peptide concentration was varied from 10^{-11} to 10^{-7} M (horizontal axis on the plots). (The units of peptide concentration are $\log_{10}$M unless otherwise marked).

- Four sets of experiments probing the CD8 effect were conducted, these are marked by the legend on the chart as:

'PBS' - incubation with cell medium alone (phosphate buffered saline), as a control;

'CD8 Ab' - incubation with an anti-CD8 monoclonal antibody, OKT8, which should have some inhibitory effect by binding to CD8 on the T cell surface and blocking the interaction between this and HLA-A2;

'CD8' - incubation in the presence of soluble CD8 receptor, at 100 µg/ml;

' CD8 mutant' - incubation in the presence of soluble CD8 receptor with the 54-55E double mutation at 100 µg/ml.

The graphs of figure 8 show the results collected after 2 hours incubation.

Figure 9 shows data from the same experiments as Chart 1 except that the data were collected after 6 hours incubation.

The data in figures 8 and 9 demonstrate:

- that more target cells are killed at high peptide concentration which is as expected. At low concentration the density of specific target molecules on the target cell surface is too low to activate the T cells.

- CD8 antibody which blocks CD8 on the T cells has some inhibitory effect compared to PBS (control).

- Soluble CD8, which must be assumed to bind the MHC on the target cells, has a clear inhibitory effect, even better than that of the CD8 antibody.

- The effect of the soluble CD8 is specifically related to its binding to MHC as the mutant which has two critical amino acids substituted has no inhibitory effect.

Figure 10 shows the results of a series of experiments conducted as follows:

- The same human T cell line recognising the HIV1 'gag' peptide restricted by HLA-A2 was used as in the experiments of Figures 8 and 9.

- A constant number of target cells and T cells, 5000 of

each were used.

- The peptide concentration was varied from 10^{-11} to 10^{-7} M (horizontal axis on the plots).The legend is marked as follows

Five sets of experiments probing the CD8 effect were conducted:

'PBS' - incubation with cell medium as a control;

'CD8 A' - incubation in the presence of one preparation of soluble CD8 receptor at 100 μg/ml;

'CD8 B' - incubation in the presence of a second preparation of soluble CD8 receptor at 100 μg/ml;

'Mutant 22' - incubation in the presence of soluble CD8 receptor with the 54-55E double mutation at 100 μg/ml;

'Mutant 15' - incubation in the presence of soluble CD8 receptor with the 99E mutation at 100 μg/ml.

Results were collected after 2 hours incubation.

The data in figure 10 extend the conclusions drawn from data in figures 8 and 9, demonstrating similar effects of two preparations of soluble CD8 compared to two mutant CD8s.

Figure 11 shows experiments were performed as described above except that the concentration of soluble CD8 was varied from 0 - 100 micrograms/ml. The three traces correspond to peptide concentrations of 10^{-9}, 10^{-8} and 10^{-7} M.

The data in figure 11 demonstrate that the CD8 inhibition of cell lysis is titratable.

Figure 12 shows data from an experiment similar to those outlined above except that a T cell clone specific for HIV1 peptide ' pol' restricted by HLA-A2 was used.

E:T ratio was 5/1, peptide concentration varied between 10^{-7} and 10^{-5} M.

'Lysis' shows the absolute readout, 'specific lysis' shows

results corrected for background.

The data in figure 12 demonstrate that the inhibitory effect of CD8 is also observed with a T cell clone.

Figure 13 is another titration of CD8 inhibition showing both target cell lysis and T cell stimulation as assessed by MIP1β production. MIP1β is a T-cell activation marker which is produced by T cells in amount proportional to their activation state. This demonstrates that the inhibitory effect is correlated to an inhibition of T cell activation.

Figure 14 shows data from experiments involving a rare T cell response that is independent of CD8. The experiment is run similarly to those previously described in that a different sample of cytotoxic T lymphocytes were incubated in a killing assay with '868' B cells at a E:T ratio of 5:1 and with varying concentrations of OKT8 CD8 antibody. The legend shows the data collected after 30 min:

'PBS' incubation with cell medium as a control

'600/100/10 μg/ml' - concentration of OKT8 CD8 antibody used in each incubation dataset

The data in figure 14 demonstrate that the CTL response for this particular sample of T cells cannot be inhibited by CD8 antibody except at extremely high concentrations, at which any protein would inhibit due to toxic effects. Thus the CTL response for this particular cell type is CD8 independent.

Figure 15 shows data from a similar experiment with the same cells incubated with soluble CD8 receptor at a range of peptide concentrations under similar conditions i.e. E:T 5:1, '868' B cells used as targets. The legend corresponds to:

'PBS" incubation with cell medium as a control

'CD8' incubation with soluble CD8αα receptor at 100 μg/ml.

The data in figure 15 demonstrate that the CTL response for this cell sample is not be inhibited by soluble CD8.

Thus the data in figures 14 and 15, taken together, demonstrate that the soluble CD8 mediated inhibition observed with the CTL responses examined in figures 8 to 13 is not due to an unspecific effect.

CLAIMS

1. A protein having the sequence as shown in figure 1, said protein folded as a dimer and having the property of inhibiting the action of cytotoxic T cell lymphocytes to kill target cells.

2. A protein which differs from that shown in figure 1 in one or more of the following respects:

- methionine present at the N-terminus;
- one or a few amino acid residues absent from the N-terminus;
- one or a few amino acid residues added at the N-terminus consisting of part or all of the sequence leu - leu - leu - his - ala - ala - arg - pro -;
- one or a few amino acid residues absent from the C-terminus;
- one or a few amino acid residues added at the C-terminus consisting of part or all of the sequence - ala - pro - arg - pro - pro -thr - pro – ala;
- part or all of the CD8 cytoplasmic membrane peptide sequence added at the C-terminus;
- conservative variants of one or many amino acid residues which do not materially affect the CD8 functionality of the protein;
- mutations which do alter the CD8 functionality, including those which increase or abolish the property of inhibiting the action of cytotoxic T cell lymphocytes to kill target cells;
- the addition of a protein or peptide, at the N or C terminus for the purposes of purification;
- the provision of a label for detection;

said protein folded as a dimer being soluble having the property of affecting the action of cytotoxic T cell lymphocytes to kill target cells.

3. A protein according to claim 2, in which E replaces N at position 99.

4. A protein according to claim 2, in which EE replaces QN at position 54, 55.

5. A complex of a protein according to any one of claims 1 to 4 with HLA - A2.

6. A complex as claimed in claim 5 in crystalline form with a stoichiometry of one protein dimer to one HLA - A2 peptide unit.

7. A method of preparing a formulation for affecting the immune system of a patient, which method comprises bringing a protein according to any one of claims 1 to 4 into a form suitable for administration.

8. A method of producing the recombinant protein according to any one of claims 1 to 4, which method comprises the steps of:

i) providing a CD8 derived gene suitably modified to allow expression of a protein, essentially corresponding to at least the immunoglobulin-like domain of a CD8 protein, in a bacterium;

ii) effecting expression of said CD8 derived gene in said bacterium and recovering of the expressed protein from a bacterial culture;

iii) treating the expressed protein to facilitate its purification and carrying out said purification.

9. The method of claim 8 wherein: at step i) the CD8 derived gene is modified via silent mutations designed to increase expression via the prevention of the formation of a 5' hairpin secondary structure in the expressed mRNA.

10. The method of claims 8 or 9 wherein: at step iii) the treatment of the expressed protein involves solubilising the protein and treating the protein so as to cause it to fold into a form resembling its native state, which is then purified.

11. The method of any one of claims 8 to 10, wherein the CD8 derived gene product corresponds to the immunoglobulin-like and membrane-proximal stalk regions of a CD8 protein.

12. Use of the protein of any one of claims 1 to 4 as a means of modulating an immune response via contacting said protein with cytotoxic T lymphocytes.

13. Use of the CD8 derived protein of claims 1-6 as a means of inhibiting the immune response of cytotoxic T lymphocytes.

FIGURES

```
          10          20          30          40          50          60
SQFRVSPLDR TWNLGETVEL KCQVLLSNPT SGCSWLFQPR GAAASPTFLL YLSQNKPKAA

          70          80          90         100         110         120
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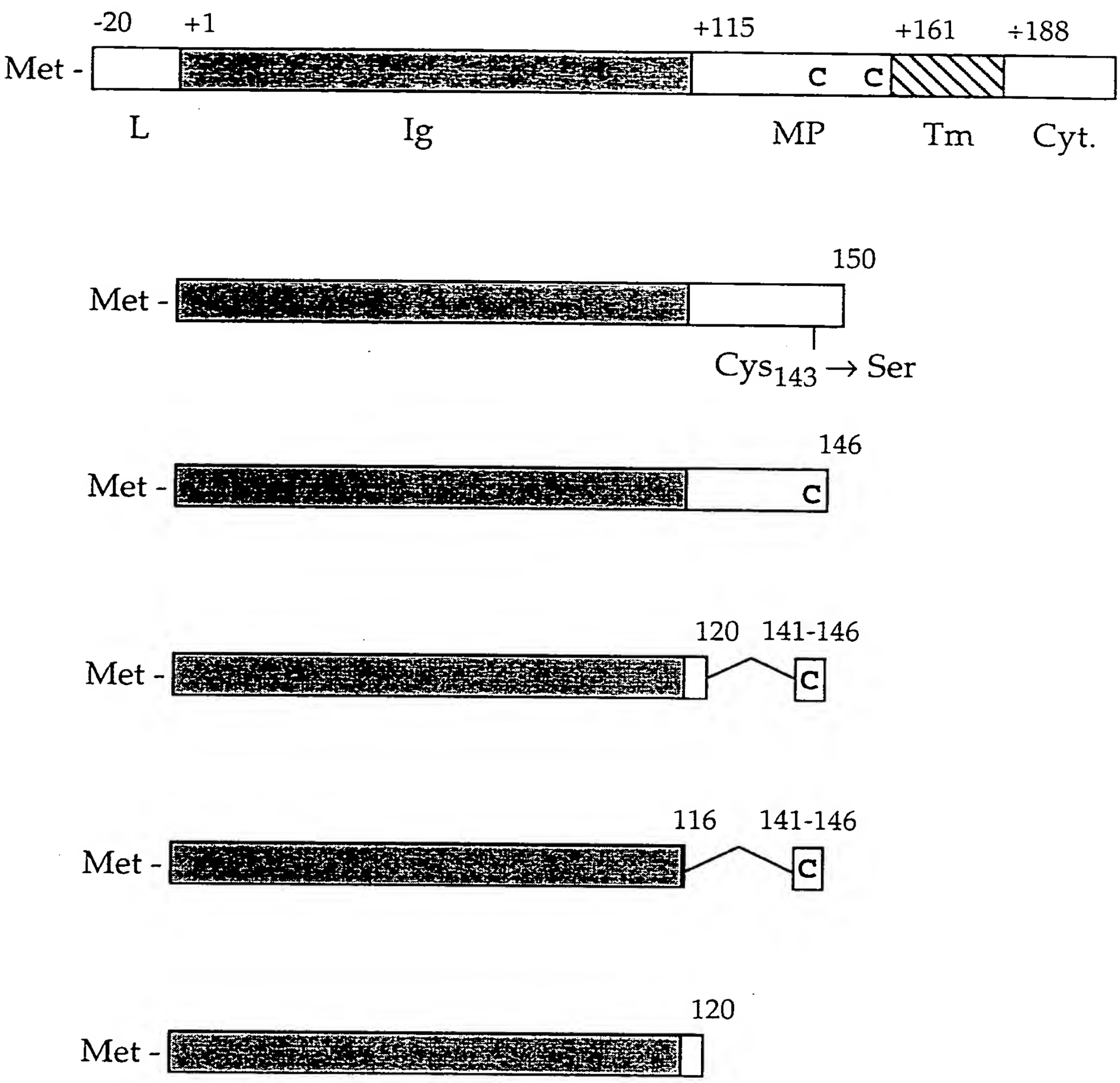
Figure 1. Modified CD8 protein as expressed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+161
÷188
Met -
C
C
L
Ig
MP
Tm
Cyt.
150
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C
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Met -

Fig 2.

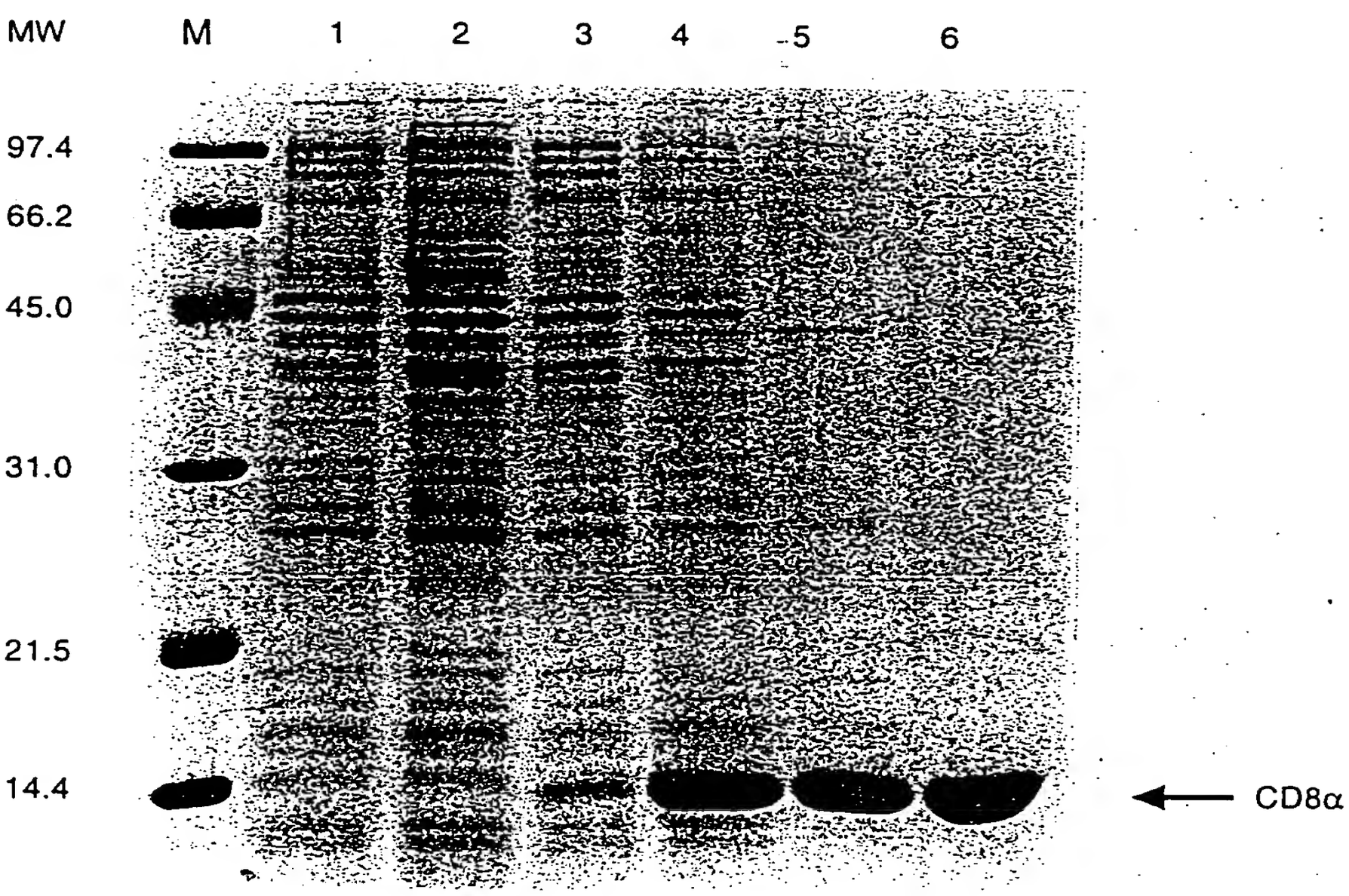
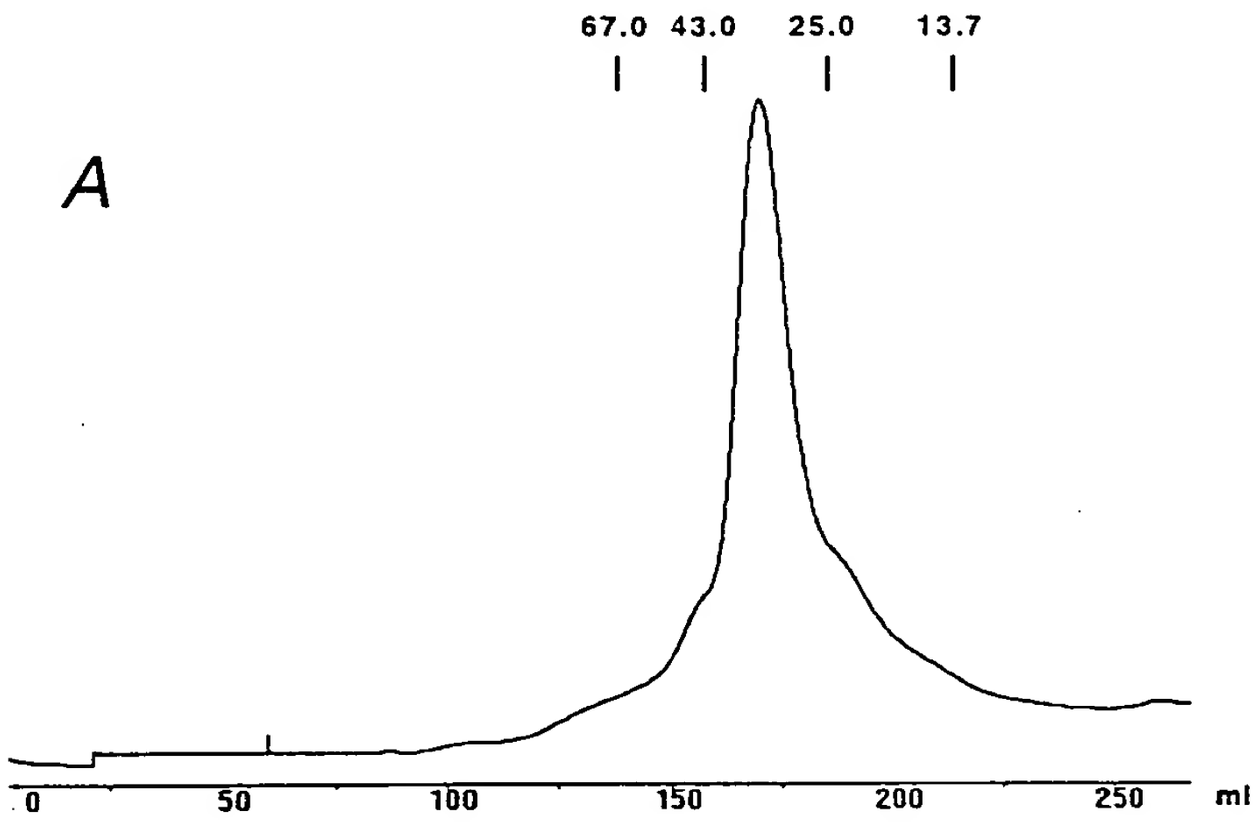


Fig. 3



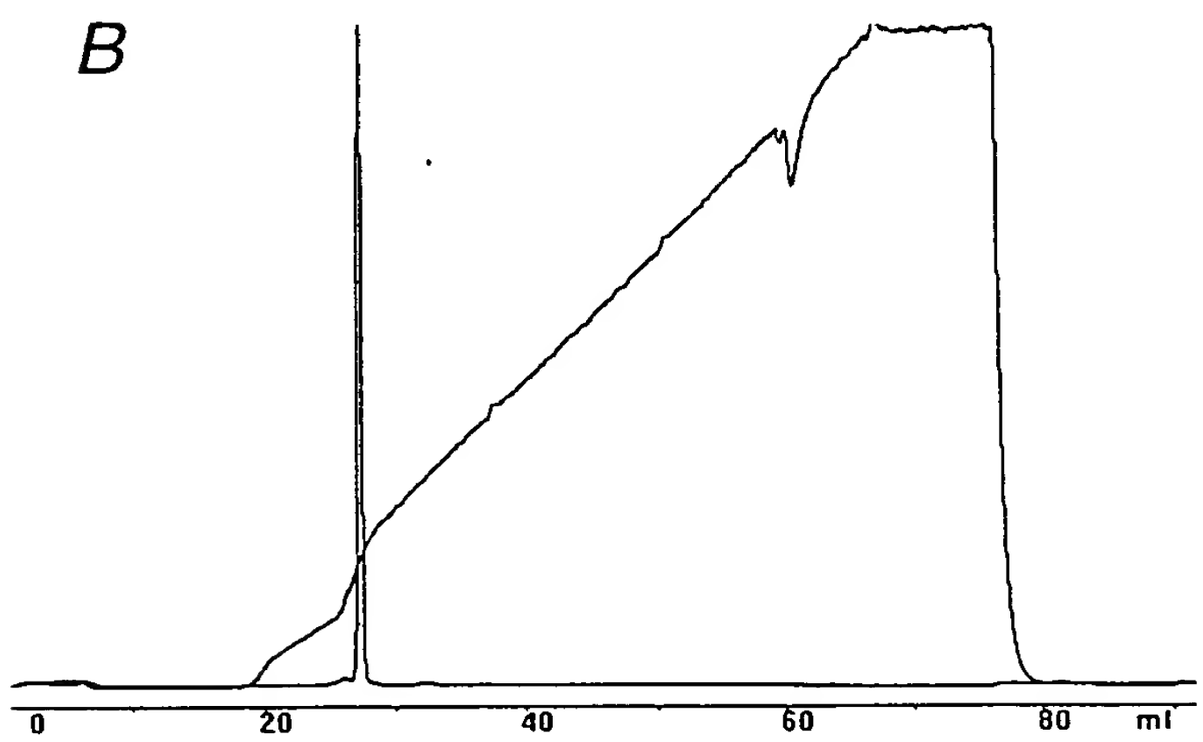
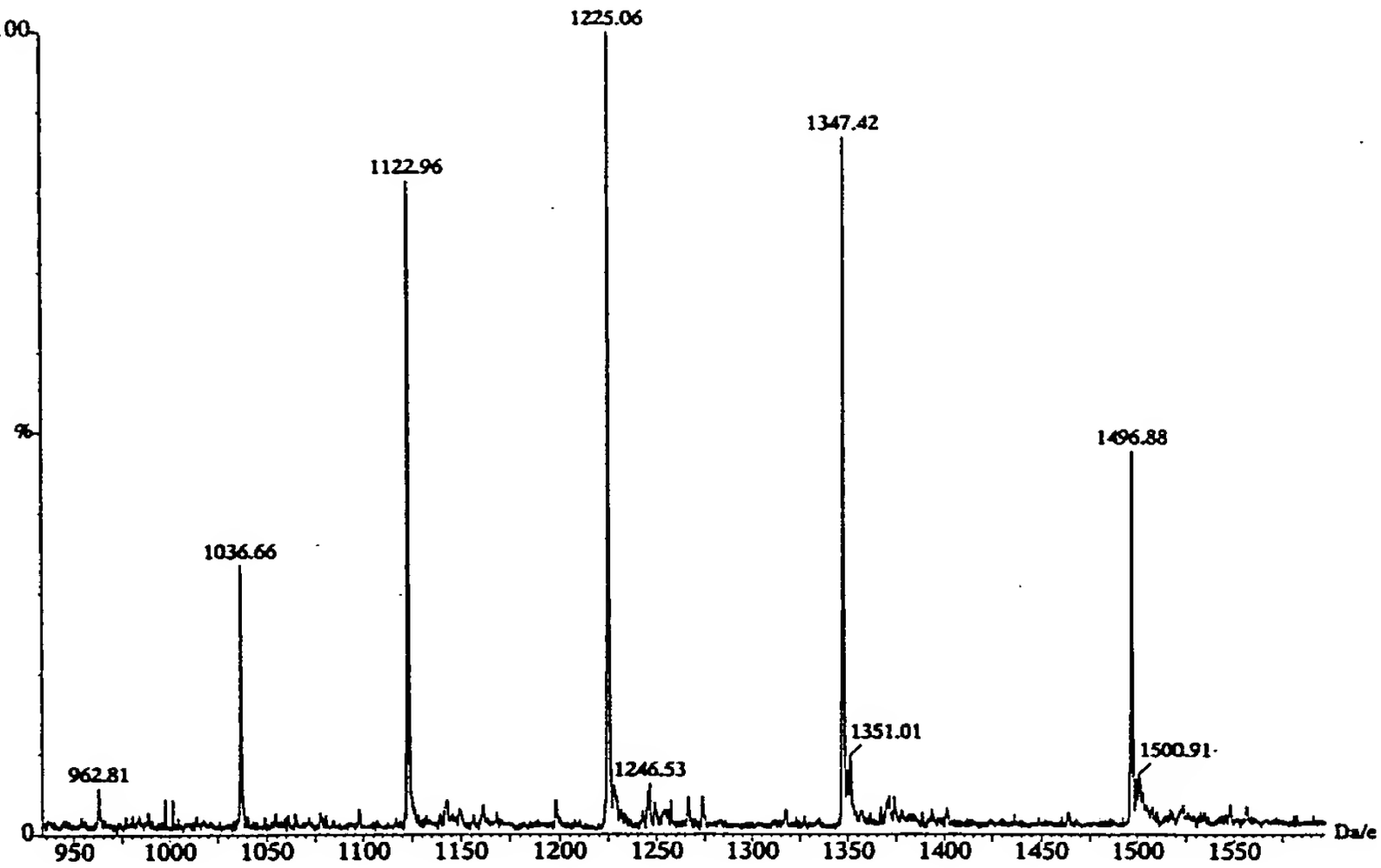


Fig 4

A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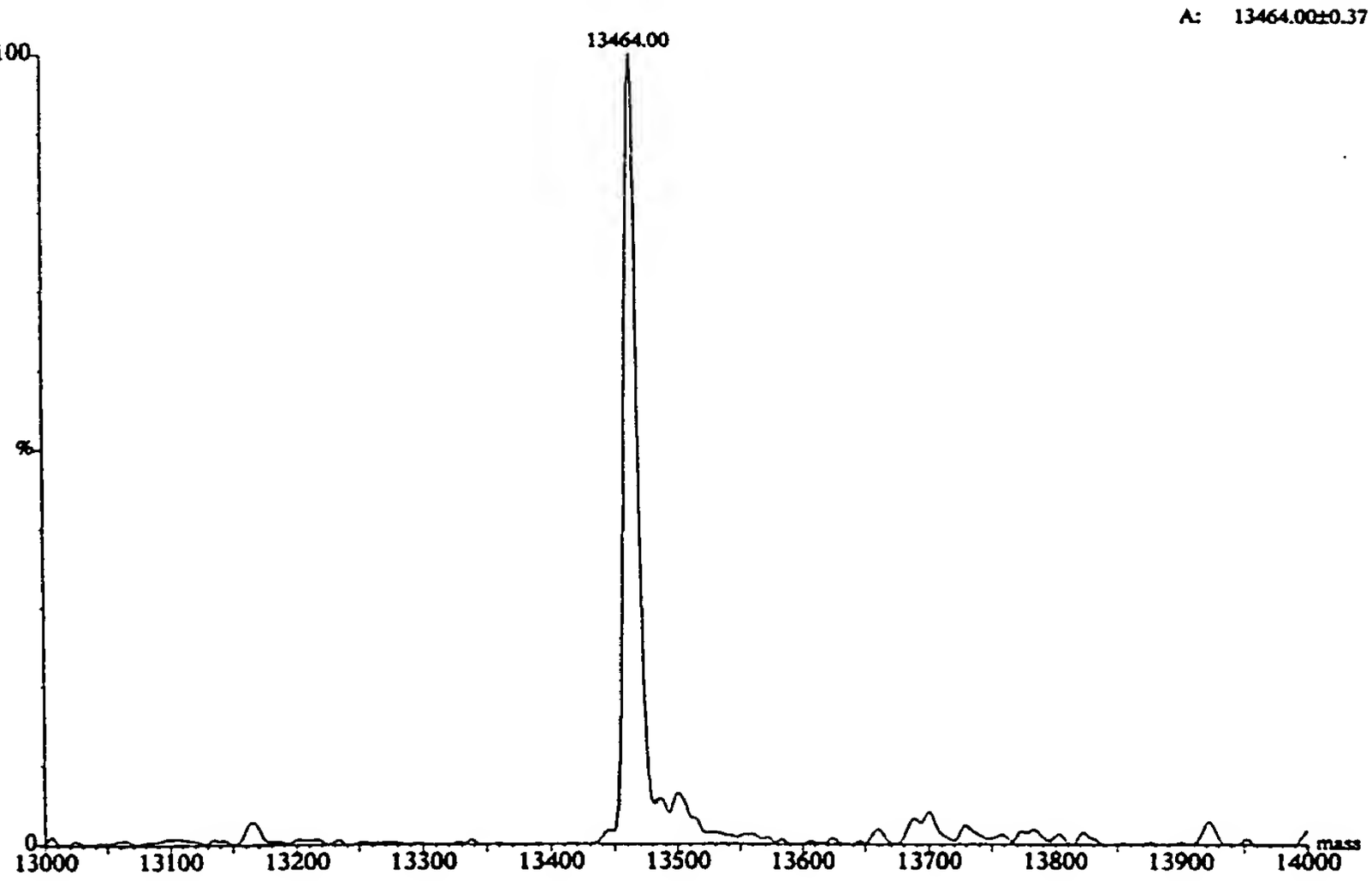
1500
1550
Da/e

B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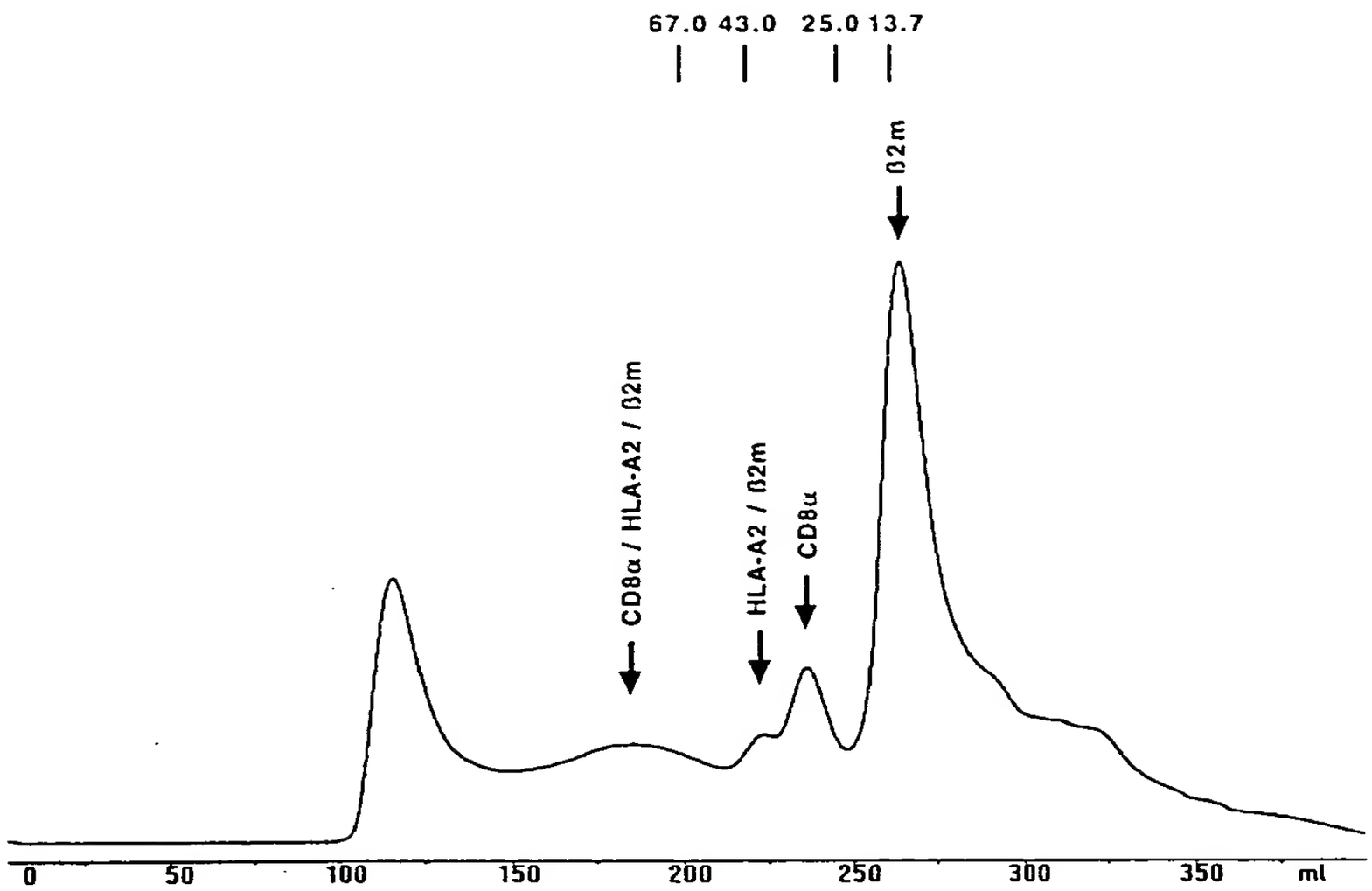
mass

Fig 5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β2m
CD8α / HLA-A2 / β2m
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200
250
300
350
ml

Fig 6

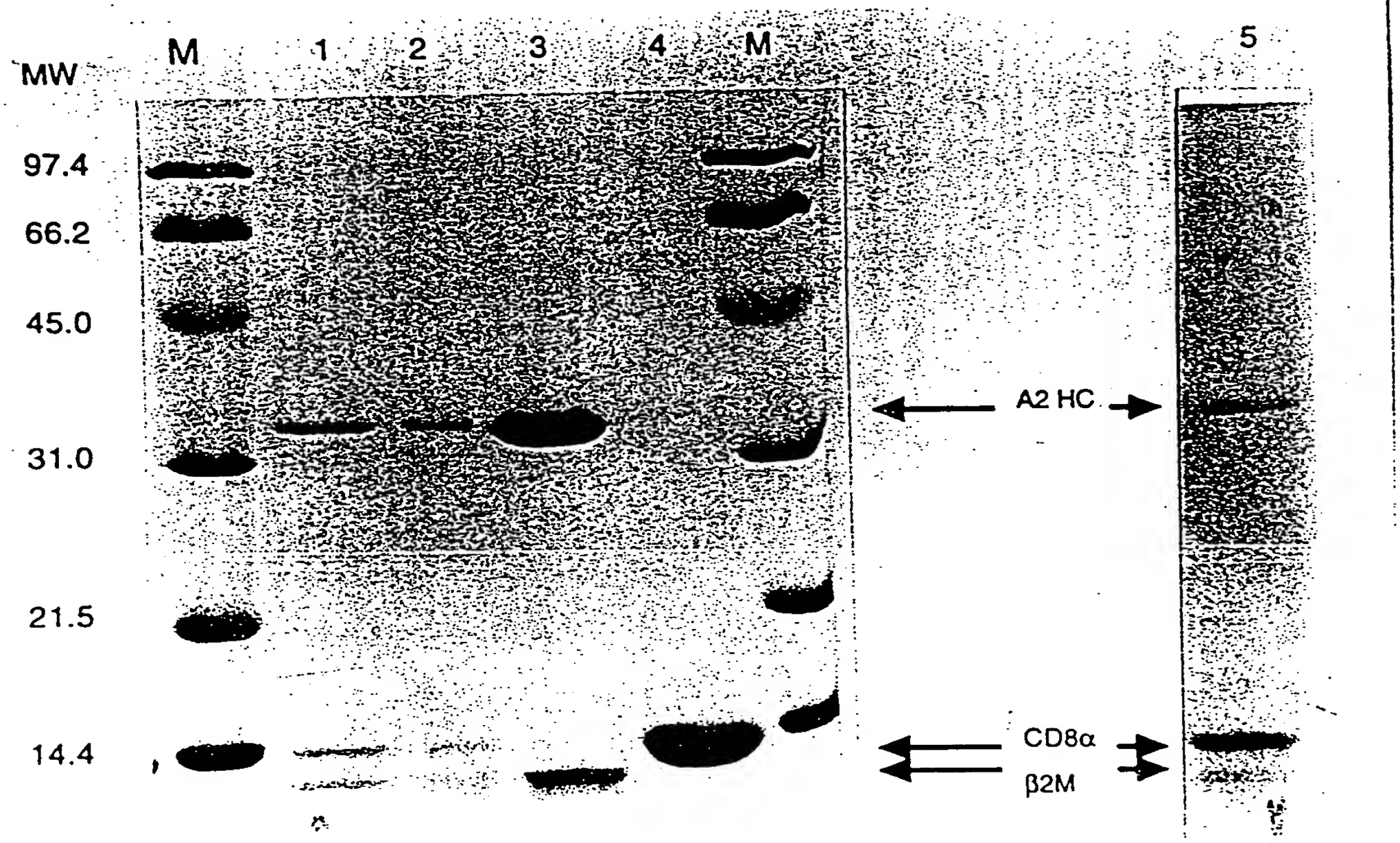
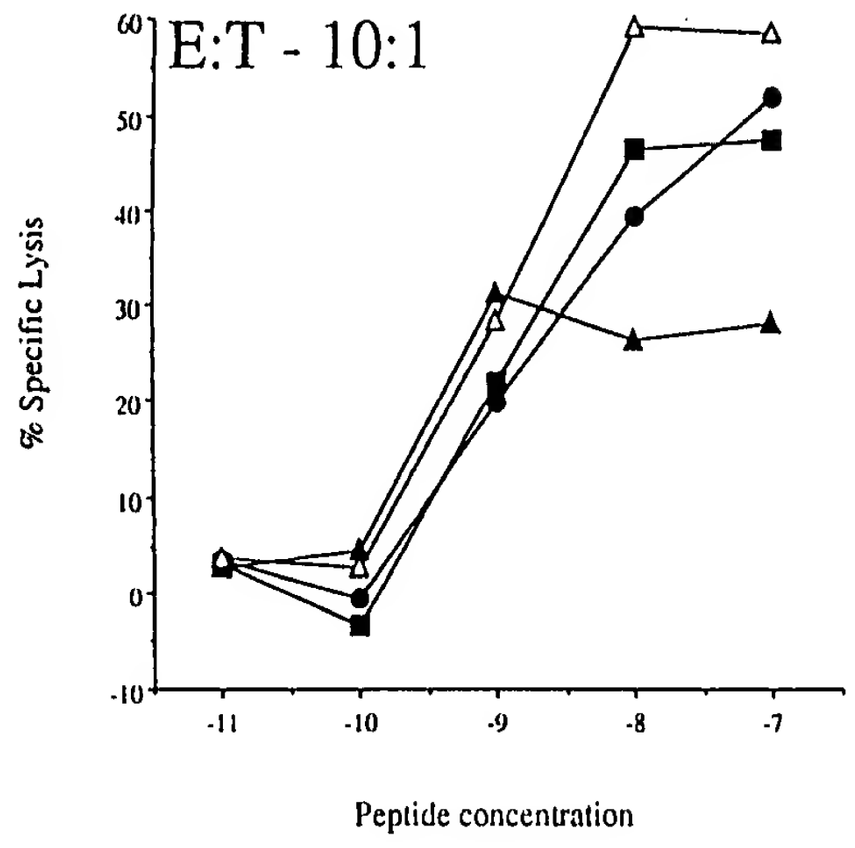
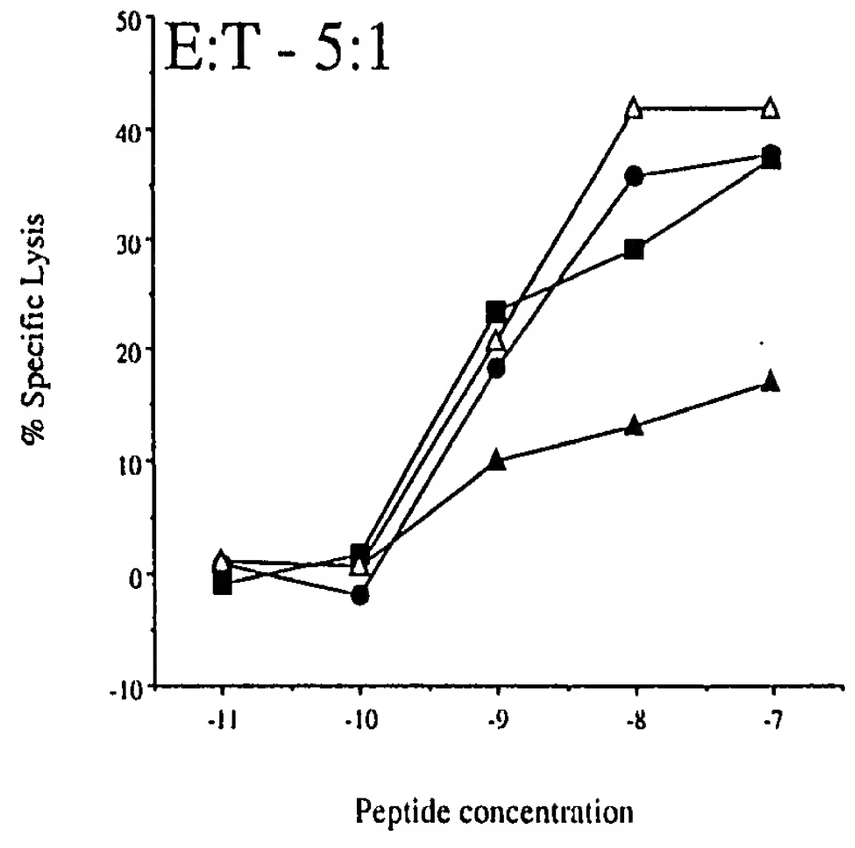
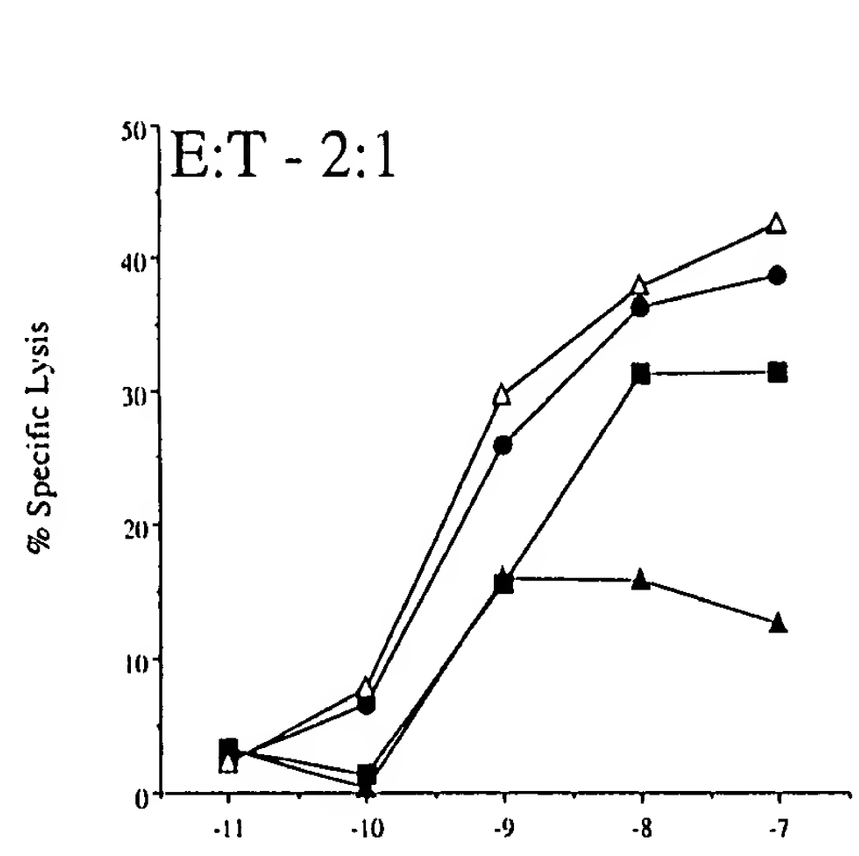


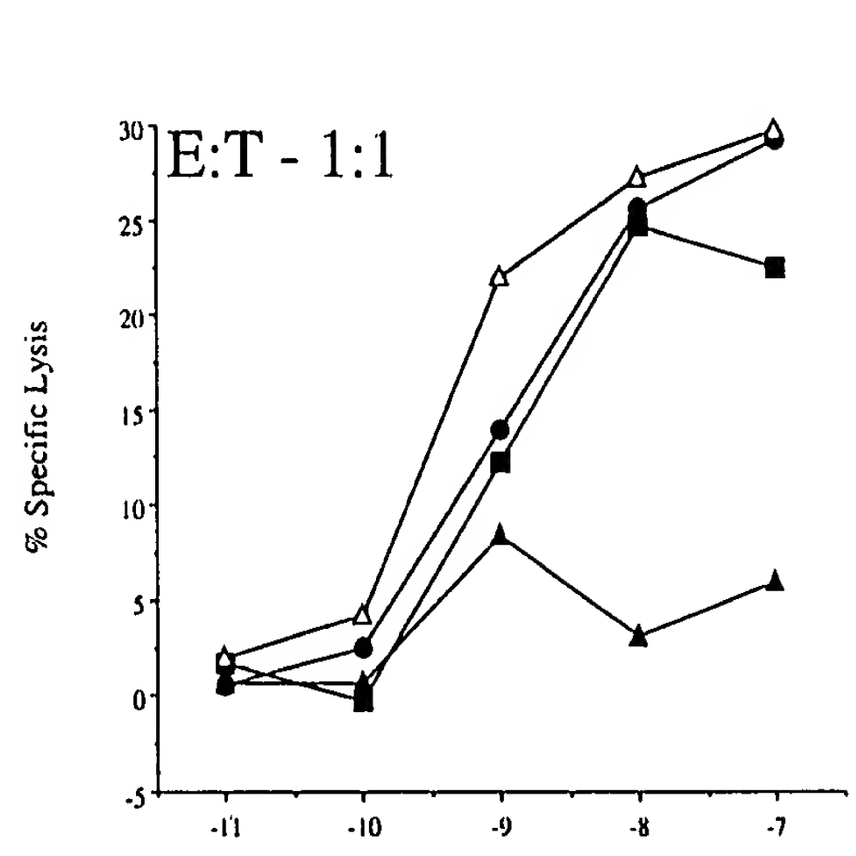
Fig 7.

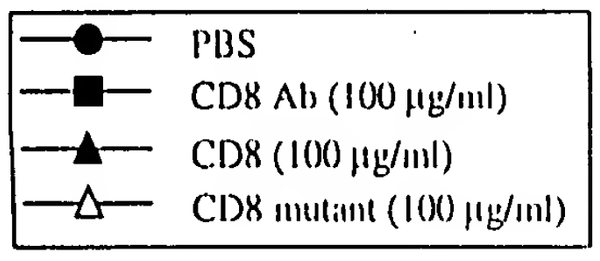
2 hour assay







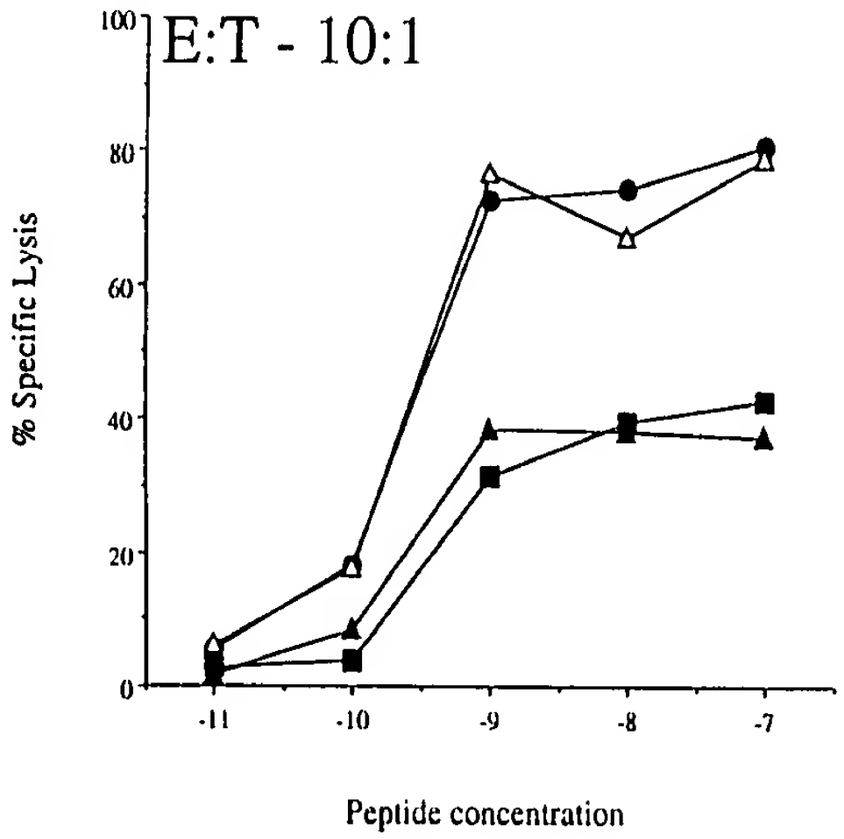


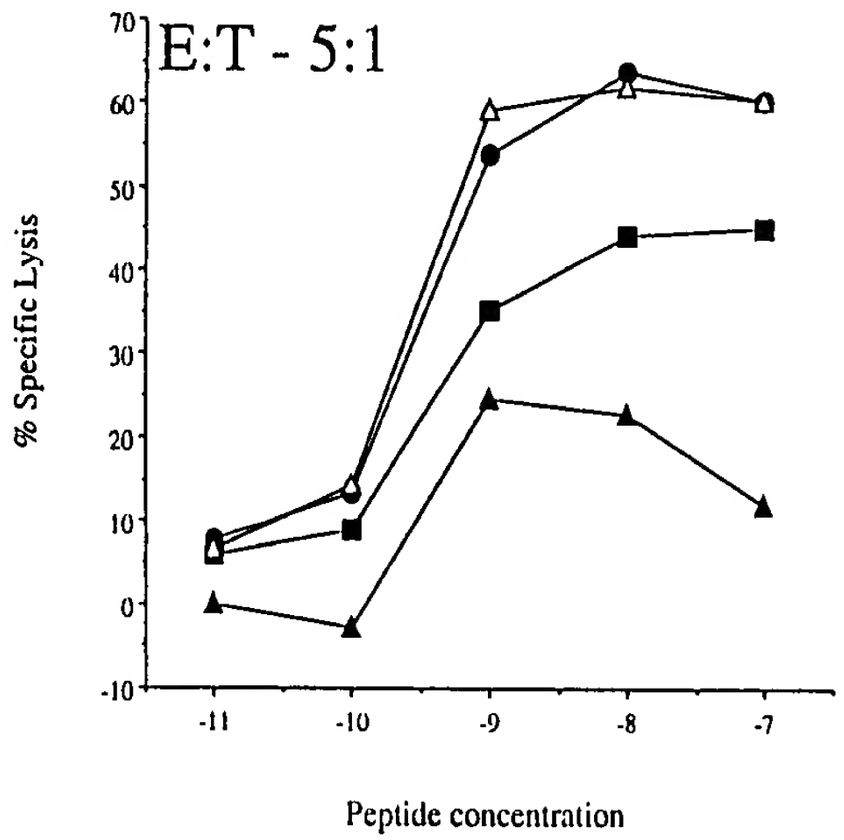


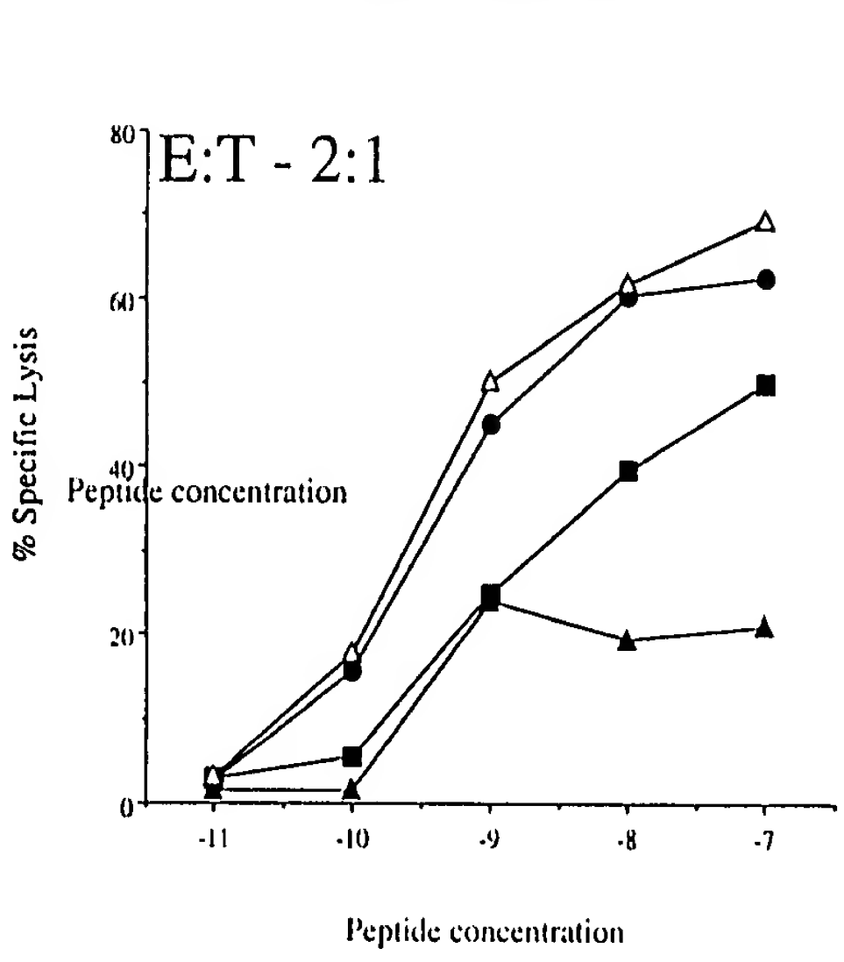
2/9/97 003 A2 Gag (SLYNTVATL) killers used.

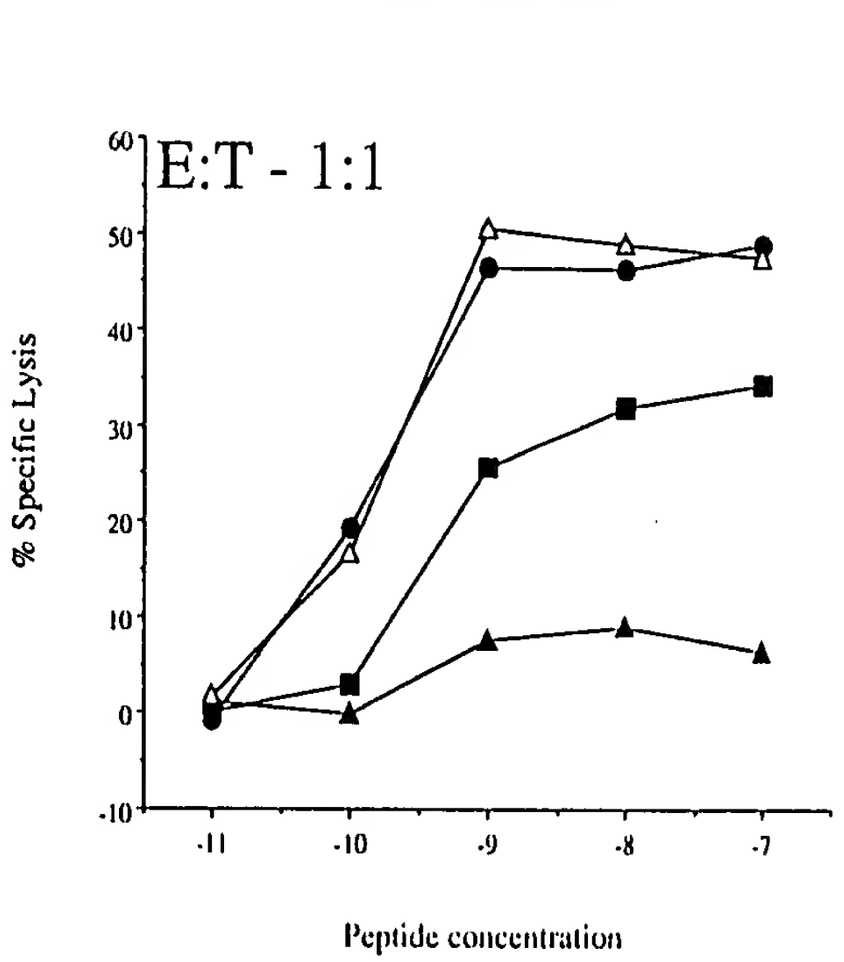
Assay: 18 µl 10X peptide
18 µl PBS or PBS with 1mg/ml CD8a/a or CD8 OKT8 Ab
50 µl Targets (5 000 868 B cells)
100 µl containing 5 000 -50 000 CTL

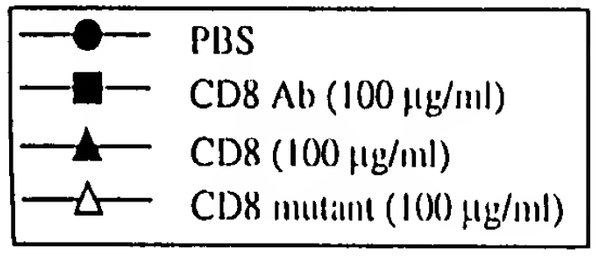
6 hour assay











2/9/97 003 A2 Gag (SLYNTVATL) killers used.

Assay: 18 µl 10X peptide
18 µl PBS or PBS with 1mg/ml CD8a/a or CD8 OKT8 Ab
50 µl Targets (5 000 868 B cells)
100 µl containing 5 000 -50 000 CTL

4/9/97 Effect of CD8 and CD8 mutants on CTL lysis
003 CTL @ E:T of 1:1. 2 hour assay

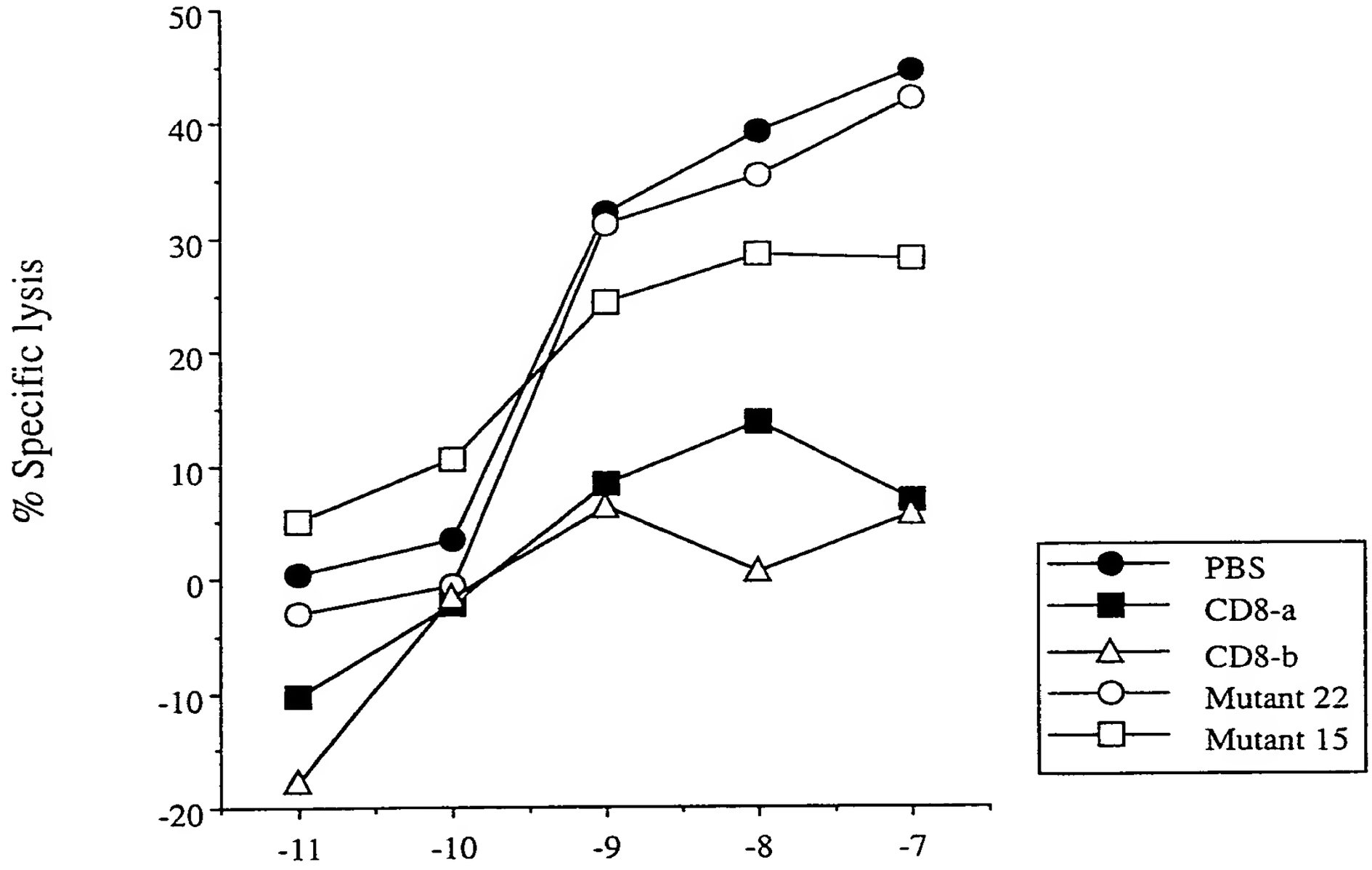


Fig 1

4/9/97. Effect of CD8 on CTL lysis
003 A2 Gag CTL. E:T - 1:1. 2 hour assay.

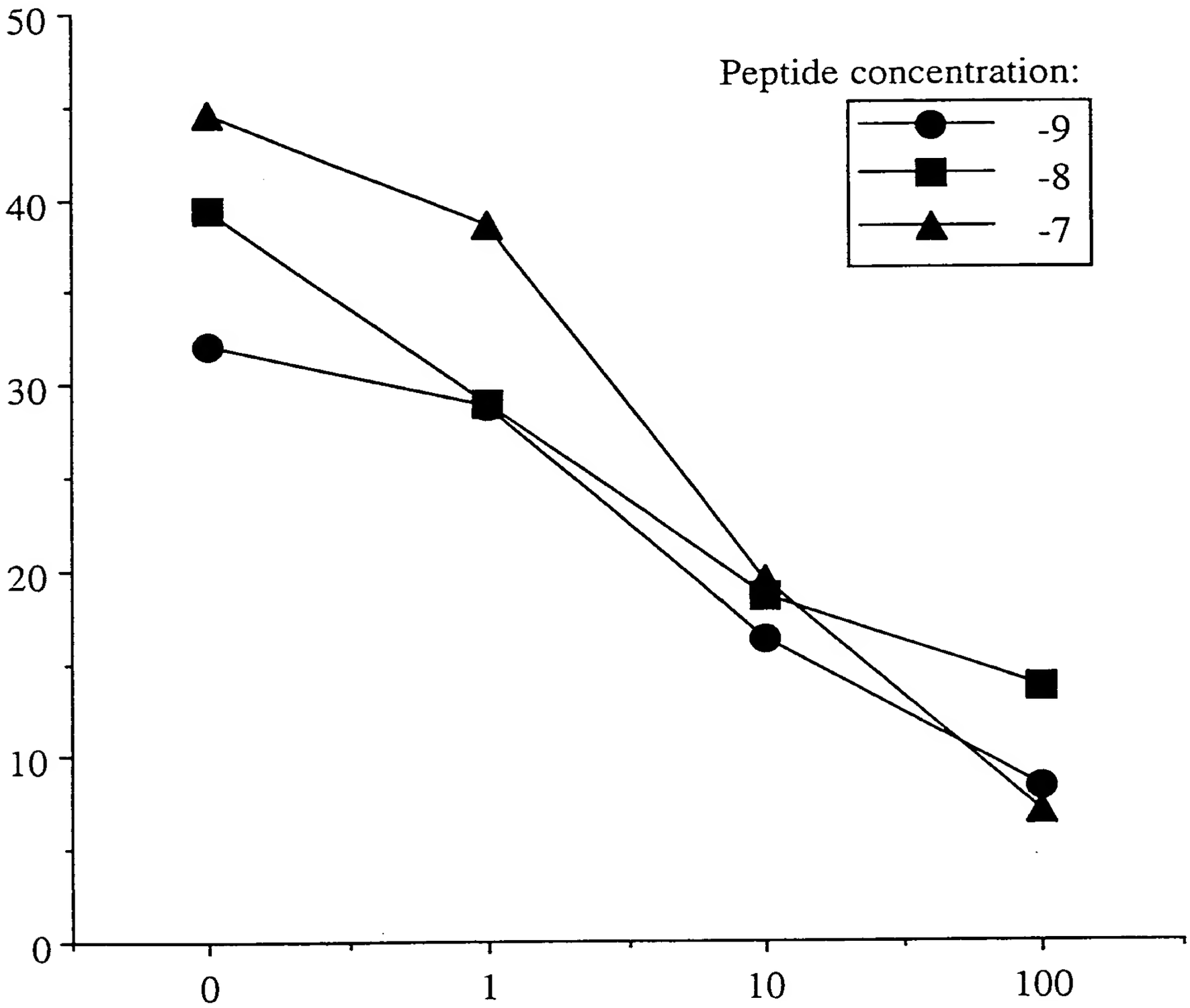
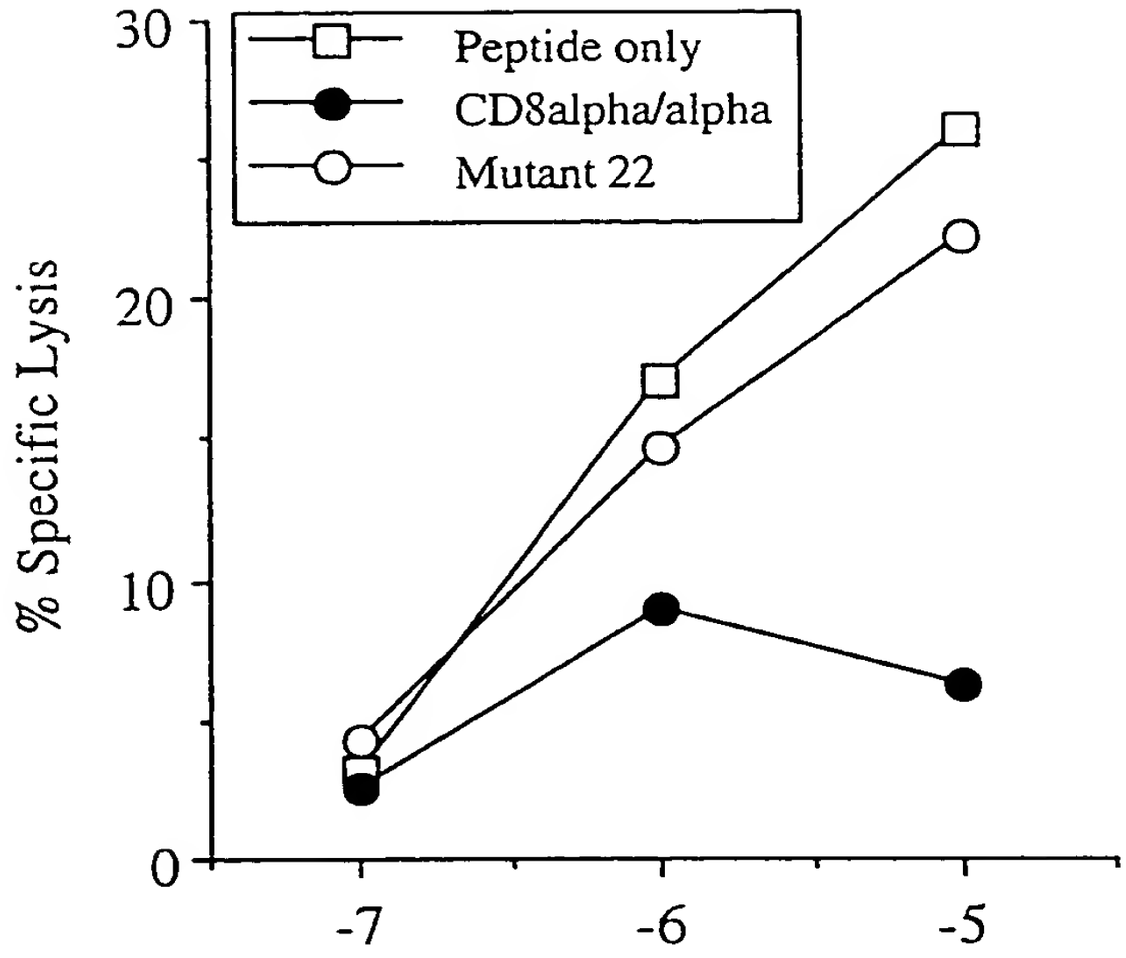


Fig 12



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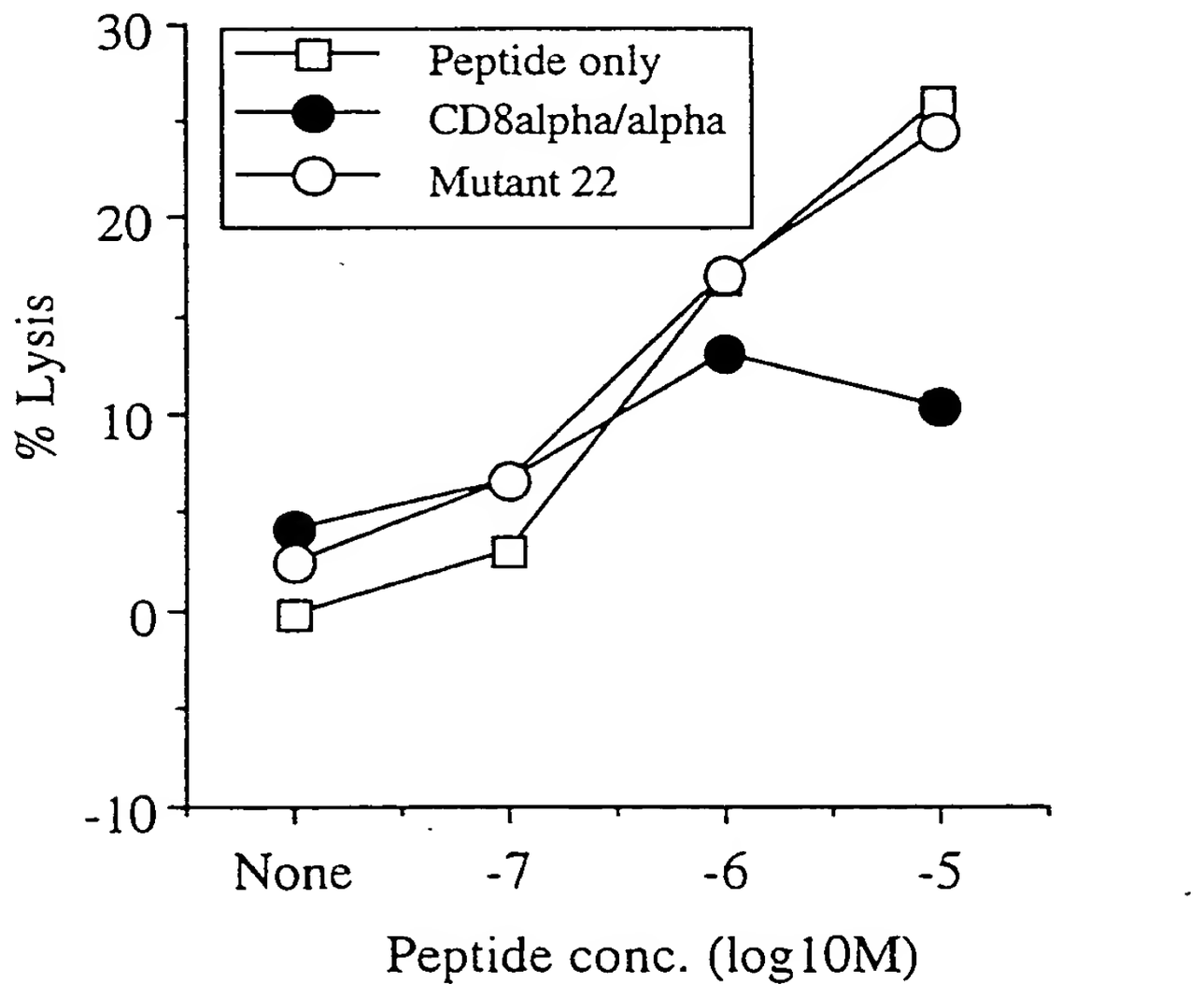
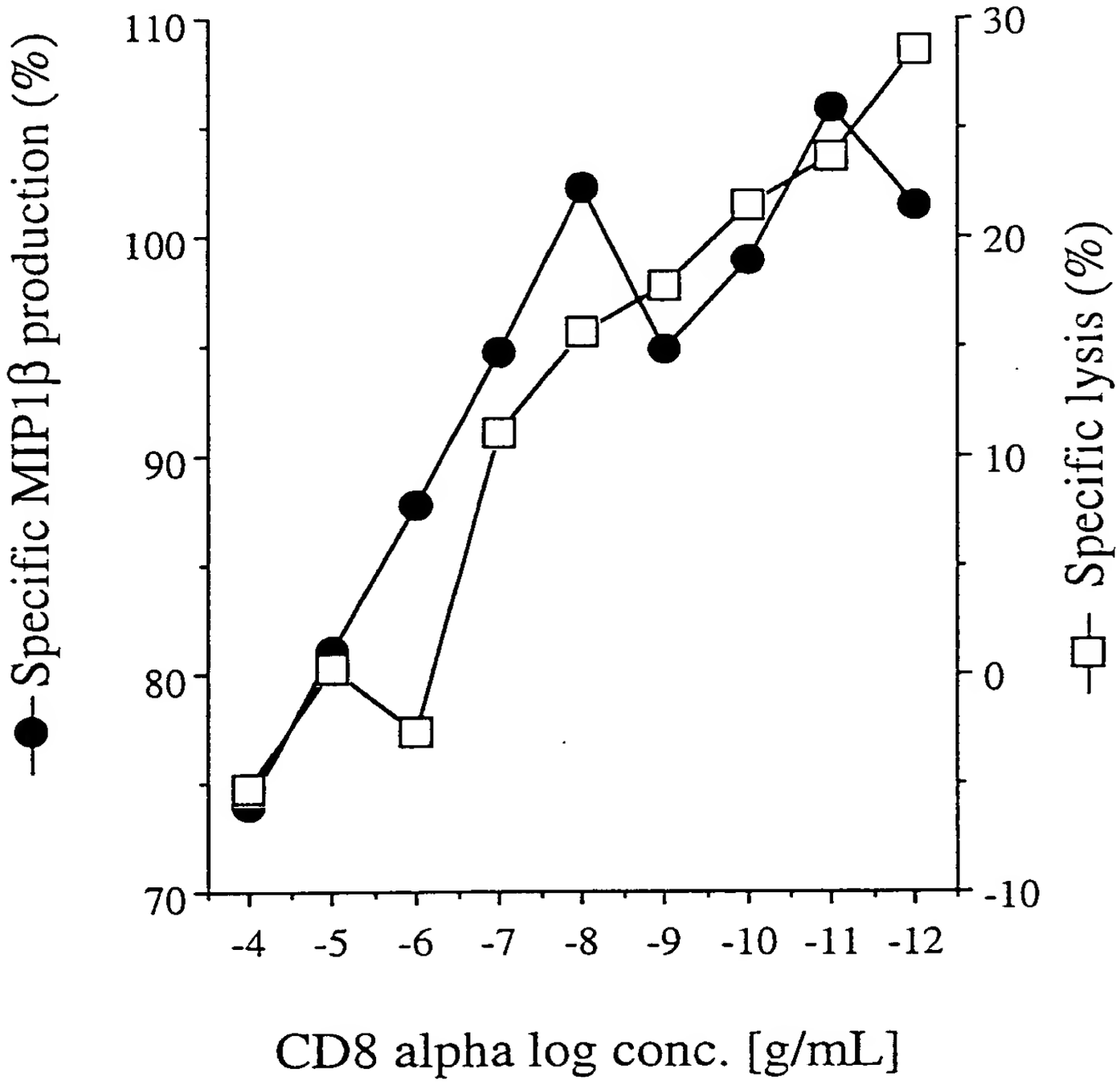
E/T : 5/1
Targets : 868 EBV-transformed BCL



CTL : HLA-A*0201 restricted Pol (VIYQYMDDL) clone 10
E/T : 5/1
Targets : 868 EBV-transformed BCL

9300868 A2 gag line



Effect of CD8α/α homodimer on lysis and MIP1β production by A2 Gag (SLYNTVATL) CTL from patient 9300868

Lysis experiment
E:T 8:1
Peptide conc. 10uM
No peptide control contained 100ug/mL CD8 alpha (shown)
Subtracted baseline is no peptide/no CD8alpha negative control

MIP experiment
10^5 cells per well
10 μM peptide used in assay of total volume 186 μl

Fig 4

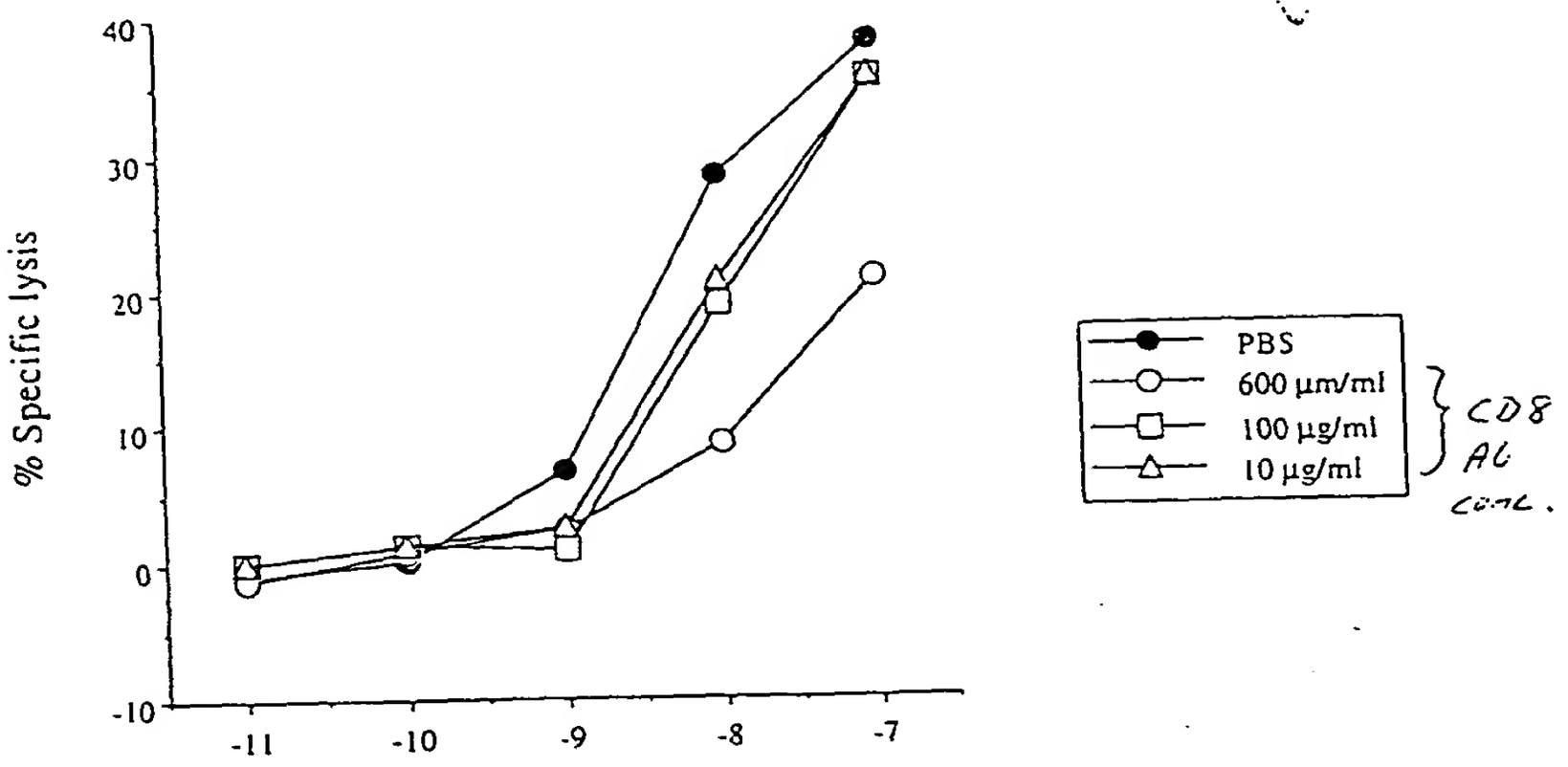
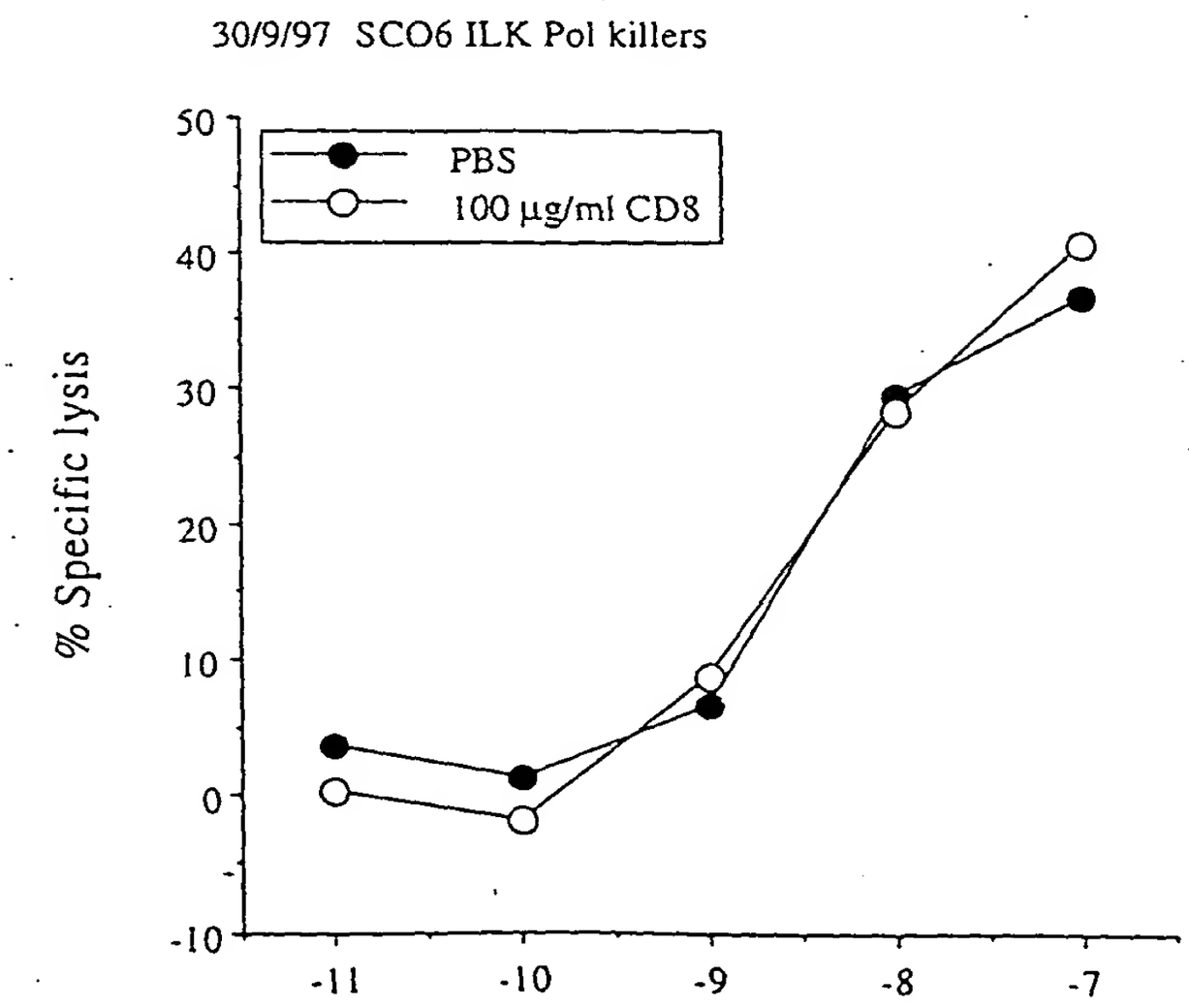

% Specific lysis
40
30
20
10
0
-10
-11
-10
-9
-8
-7
Peptide concentration
PBS
600 µm/ml
100 µg/ml
10 µg/ml
CD8
AL
CONC.

Fig 5


30/9/97 SCO6 ILK Pol killers
% Specific lysis
50
40
30
20
10
0
-10
-11
-10
-9
-8
-7
PBS
100 µg/ml CD8